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Winter

Spring

History of Pharmacy as an Academic Discipline
GEORGE URDANG*

A NEW ERA within the teaching of the history of pharmacy is to be inaugurated by this lecture. An era in which the history of the scientific and professional activities that we think belong to the sphere of what we call pharmacy will be taught and studied not as a sideline any more, but in an analogous way and under the same conditions as the other branches of the history of the sciences and professions and even, for the time being, in advance of some of them.

Pharmacy has had to go a long way to achieve this goal and it is primarily this way, the history of the history of pharmacy so to say, to which this introductory lecture will be devoted.

There is one question which has to be answered in the very beginning. Have the concepts "pharmacy" and "history" been definitely fixed at a more or less early time or have they too, like the subjects they have been dealing with, paid their tribute to the changes within a continuously changing world? As was to be expected they have done the latter. They have undergone changes in the course of time.

There was a time when the pharmaceutical activities, the sum total of which we call pharmacy, were an integral part, and a very important one, of the art of the physician. It was followed by a time when these activities, expanded as they were by the continuous additions to the *materia medica* and more complicated methods of preparation and drug storage, had become the task or at least responsibility of a new group of experts, the professional pharmacists. It is understood that, at this time, the term "pharmacy" was used mainly as a designation of the work done and the part played by these people who were supposed to cover the whole field, the pharmaceutical retail business as well as the manufacturing of complex drugs.

At the height of this period the young science of chemistry found its promotion, its places of work, and the men to do the work in the labora-

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tories of these pharmacists to such an extent that the great historian of chemistry, Hermann Kopp, did not hesitate to call pharmacy the mother of scientific chemistry. This flattering statement, made just a century ago, has given rise to the notion that in those days pharmacy and chemistry were so closely connected that their history during this period is almost identical. Although this idea of identity goes too far, there cannot be any doubt that a presentation of the History of Chemistry which should attempt to bypass or minimize the part of pharmacy in the development of chemistry would be quite as incomplete as would a History of Pharmacy similarly minimizing chemistry and its meaning to pharmacy.

With the rapid growth of chemistry in all its branches and ramifications, creating as well as subjected to a definite change in economic world conditions based on and requiring mass production, pharmacy experienced a division of labor changing its entire structure. The various tasks to be performed by pharmacy have been gradually taken over by special groups of people. Still retail pharmacy with its twin sister, hospital pharmacy, represents the hub of the wheel, offering the place at or from which the individual customer is furnished with the drugs needed. The manufacturing part, however, has become the task of a special industry growing steadily in importance and assigning to retail pharmacy a new responsibility, that of adviser to the medical practitioner as well as to the public with regard to the nature of modern remedies. There has developed furthermore an organized wholesale trade in drugs and accessories conducted by or using the services of pharmacists. Hence it is not retail pharmacy and its activities alone any more, but the sum total, the coordinated endeavor of all the groups participating in the tasks belonging to or connected with the meeting of the medicinal needs of the people everywhere, which is now designated by the term "pharmacy," and which must be made subject to the history of pharmacy.

And what about the concept of "history"? There was a time when history was regarded as a narrative or a chronological account of data and dates centering around the fate of dynasties, of individual kings and heroes and the endless chain of wars. Gradually the point of view has changed. The general interest has shifted from royalty to the plebs, to the common people and their fate, and from the mere recording of facts to attempts at their adequate interpretation. We want to know not only the what but, as far as possible, the why also. The fact-finding has to be followed by an attempt at sense-finding.

As for pharmacy, we do not think it sufficient any more to know a number of details about the profession, its tasks, its work, and fate throughout the ages. We have become aware of the fact that nothing is isolated in this world of ours, that whatever has happened, and is going to happen, in or to pharmacy reflects the general situation of the time concerned, and we wish to know about this interrelationship in order to understand both the development of the general situation and that of pharmacy.

It has been said before that pharmacy, although it has never been identical with chemistry, has always been closely connected with this most important of its basic sciences. This connection has been based on the substantial similarity of the work done in the pharmaceutical as well as in the chemical laboratory. And as was pointed out before, there has existed since time immemorial a still closer connection between pharmacy and medicine. This connection, however, and this is a very important fact, has been one of a common purpose, that of preventing and curing disease, rather than a relationship based on any substantial analogy to the work expected of the two professions for the sake of society.

Whenever a wrong interpretation of this common purpose or other circumstances have led to the concept of such a work analogy and its realization on the one side or the other, to counter prescribing by the pharmacist and/or to dispensing by the physician, it has meant a transgression and an interference which, although they might sometimes have been required by temporary necessities, have never had any real justification. It is understood that the development of the relations between medicine and pharmacy must be dealt with by the pharmaceutical historian; and in the history of drug therapy the historians of both professions have a broad field in common in which they very fortunately supplement each other.

Pharmacy and medicine, by the very nature of their tasks, have placed upon their adepts a special social responsibility, and society has, through the ages, rewarded them with a rather privileged position within the community. The members of both professions have always been typical representatives of the educated middle classes of their time, bearers and promoters of cultural standards and tendencies, and have been so held. That condition has found its expression in literature and art as well as in the part played by the members of both professions in society at large, and these relations of pharmacy not only to science but to gen-

eral culture are naturally subjects to be dealt with by the historian of pharmacy.

The field is very wide, indeed, and there is still much to be done before even a modest degree of completeness or perfection can be claimed. On the other hand, pharmacy can justly be proud of the work of love done through the ages by members of the profession, and partly by others, in the field of its history.

Since the beginning of the eighteenth century more or less elaborate sketches on the historical development of pharmacy were published in, or as introductions to, dispensatories and textbooks. To Spain goes the credit for having published the first fairly comprehensive general treatises devoted to the History of Pharmacy. However, the *Histoire des apothicaires chez les principaux peuples du monde,* by the French physician, A. Philippe, world-wide in scope as it was, may be regarded as the cornerstone of the edifice called History of Pharmacy. It was published in 1853. Of equal if not even greater importance was the *Geschichte der Pharmazie* by the German pharmacist Hermann Schelenz published in 1905 and likewise world-wide in scope. On the history of drugs enormous material has been presented by Flückiger and Hanbury, by Alexander Tschirch, and, in this country, by John Uri Lloyd.

Since the publication of the book of Schelenz, treatises on the History of Pharmacy have appeared in almost all countries of the civilized world. Only a few of those written in English since the beginning of this century may be mentioned: A. C. Wootton, *Chronicles of Pharmacy* (London 1910), Ch. H. LaWall, *Four Thousand Years of Pharmacy* (Philadelphia 1927), C. J. S. Thompson, *The Mystery and Art of the Apothecary* (Philadelphia 1940), E. Kremers and G. Urdang, *History of Pharmacy* (Philadelphia 1940), G. Urdang, *Pharmacy's Part in Society* (Madison, Wis. 1946). The number of articles of pharmaceutico-historical interest, published in periodicals and partly given more ample distribution and greater permanence by reprints, can hardly be counted.

In spite of all this endeavor, it took quite a while before the history of pharmacy found official recognition even within the profession. It was in this country that for the first time a national pharmaceutical body, the American Pharmaceutical Association, made the History of Pharmacy a part of its responsibility. The former Director of the School of Pharmacy of the University of Wisconsin, Dr. Edward Kremers, inaugurated this remarkable event. On his instigation the American Pharmaceutical Asso-

ciation created in 1902 a "Committee on the History of Pharmacy" which in 1904 was made a "Section on Historical Pharmacy," and of which the initiator was the chairman until, in 1913, he refused to accept re-election. Of course Kremers' historical inclination also found its expression in the School of which he was Director.

At about the same time that the American Pharmaceutical Association Committee on the History of Pharmacy was established, Kremers started in his home a kind of seminar on the history of pharmacy as well as of chemistry. In the catalogue for 1907-08 of the University of Wisconsin, History of Pharmacy as well as History of Chemistry appear for the first time officially as recognized subjects of instruction, both announced by the same teacher, Edward Kremers. While he surrendered in 1920 the course in the History of Chemistry to Dr. Kahlenberg, then Chairman of the Chemistry Department, he continued teaching History of Pharmacy until, some time after his retirement, the course was taken over by Dr. Richtmann.

It is noticeable that in all these years, from 1907 until the present, there has never been an interruption in the teaching of pharmaceutico-historical subjects at the School of Pharmacy of the University of Wisconsin. There certainly are only a very few, if any, pharmaceutical institutions of learning in this country which can equal this record.

And yet, Dr. Kremers knew quite well that the subject could be done full justice only if someone was enabled to devote his whole time and work exclusively to the history of pharmacy. This was certainly in his mind when, in 1939, he had me come to Madison and tried whatever he could to keep me here after my original job, the writing of the Kremers-Urdang *History of Pharmacy*, had been completed. Thus he heartily welcomed it when, early in 1941, about half a year before he died, his successor, the present Director of the School of Pharmacy of the University of Wisconsin, Dr. Arthur H. Uhl, initiated the founding of the American Institute of the History of Pharmacy with me as Director. It may well be said that this Institute has developed to what it was intended to be: a center for pharmaceutico-historical research and information.

In the years since the founding of the Institute the consciousness of history in this country has grown by leaps and bounds. Going through, actively and passively, one of the most decisive periods of world history, everybody is pondering why all this had to happen. The fact has become obvious that there is quite an incongruity between our civilization and

our culture, the enormous advances of science and their use for the eleva-
tion of our standards of life on the one side and our incapacity for coping
with these advances for the sake of a standing order of life on the other.
The idea has taken root that a better understanding of the development
and achievements of the sciences and professions might be of some help
in acquiring a greater human certainty, firmness, and determination.
For this reason the study of the history of science, and of the professions
whose tasks are based on the application of science, has been given in the
past years more attention than ever before.

The University of Wisconsin had established a chair for the history
of science in 1942, one year after the founding of the American Institute
of the History of Pharmacy. Its occupant, Professor Guerlac, left the
campus after only two years in order to do war work. He was replaced
in the fall of 1946 by Professor Stauffer, who was joined one year later by
Professor Clagett. In January of 1947 Professor Ackerknecht started
a course in the History of Medicine in the School of Medicine of the
University of Wisconsin. With pharmacy added to this team in the fall
of 1947, and considering the fact that Professor Ihde already some time
ago has started a course in the History of Chemistry which will in all
probability likewise become one of the regular teaching subjects on this
campus, the University of Wisconsin is offering to its students a unique
help in the urgent task of bridging the above-mentioned gap between
civilization and culture.

Reporting on the work of the American Institute of the History of
Pharmacy, I have for years concluded with a remark which is equally
valid for this academic course. It reads as follows:

"The aim is to equip the pharmacist for citizenship in the world of
intellectual and moral responsibility by making him familiar with the
non-technical aspects and humanistic ramifications of his profession, and
to do pharmacy's share in the cooperative endeavor for making the his-
torical record of world civilization and culture as complete as possible."

Biography of Dr. Johann Peter Frank

Imperial and Royal Court Councillor, Hospital Director and Professor of Practical Medicine at the University in Vienna, Member of various learned Societies.

WRITTEN BY HIMSELF*

Translated from the German, with an Introduction and notes, by

GEORGE ROSEN

INTRODUCTION

1745—A critical year in the annals of England! Hard on the heels of the defeat inflicted by Maurice de Saxe upon the combined English and Dutch forces at Fontenoy followed the Jacobite rebellion led by Bonnie Prince Charlie. 1745—A victorious year for Prussia! At Hohenfriedberg and Kesseldorf, Friedrich II of Prussia defeated the Austrians and brought to a victorious conclusion the Second Silesian War. And it was also in 1745, a year replete with the alarums and excursions of war that Johann Peter Frank was born. For the most part, however, his life was not affected by the storms of war, and throughout his entire career he was concerned only with works of peace and the welfare of his fellowmen.

Johann Peter Frank is best known at present as a pioneer in public health and social medicine, and he fully merits this description. Among his contemporaries, however, Frank's reputation was based largely on his activities as clinician, medical educator, and hospital administrator, and this reputation was, indeed, well deserved. For example, he opposed the separation of medicine and surgery, insisted on the need for bringing the medical student to the bedside, and emphasized the importance of establishing as early as possible within the student's mind the relations between preclinical and clinical subjects.

The most important source for Frank's life is his autobiography. It was published in 1802 at Vienna, and deals with his activities up to December 24, 1801, that is, when he was 57 years old. Frank died at Vienna on April 24, 1821. Unfortunately, he never continued the story of his life to cover this later period, but it is available from other sources.

* The autobiography, of which this title is an exact translation, was published in Vienna by Karl Schaumburg & Co. in 1802. The copy used in the translation is in the Library of the New York Academy of Medicine.

From 1795 to 1804, he was professor at the medical school of Vienna and di-
rector of the Allgemeines Krankenhaus. In 1804, however, disgusted by the enmity
and intrigues of opponents, Frank left Vienna for a post as professor of clinical
medicine at the University of Vilna. After only one year in Vilna, he was called to
St. Petersburg to become physician-in-ordinary to the Czar and director of the first
Medico-Chirurgical Academy with a salary of 42,000 rubles. After three years in
St. Petersburg, Frank was attacked by dysentery and went to Moscow for treatment.
Following his recovery, he decided to return to Vienna, but before he set out on
the homeward journey the Russian government purchased his valuable library for
20,000 rubles and Czar Alexander I bestowed upon him an annual pension of
3,000 rubles.

In 1809, Frank returned to Vienna with the intention of settling eventually in
Freiburg im Breisgau. But the outbreak of war between France and Austria com-
pelled him to stay in Vienna. On two occasions during this campaign, Napoleon
consulted Frank and invited him to come to Paris, but Frank very tactfully declined
the invitation.

On October 14, 1809, peace was signed at Vienna between France and Austria,
and Frank moved to Freiburg. However, even this beautiful city was unable to
hold him long, for in 1811 he was already on his way back to Vienna. It may be
that the death of a daughter during his stay in Freiburg led to this decision. At any
rate, Frank spent the remainder of his life in Vienna, where he practised as a con-
sultant and completed his monumental *Medicinische Polizey*. He died six years
after the Congress of Vienna.

The autobiography of Johann Peter Frank is an historical document of impor-
tance on two grounds. In the first place, as we have already pointed out, it is the basic
source for our knowledge of Frank's life and work. In the second place, however,
its significance lies in the contribution that it makes to the social history of the
eighteenth century. Frank is a representative of the Enlightenment, especially of
that version known as the philosophy of enlightened despotism. He spent his life
in the service of various rulers, great and small, and from his story one learns what
it meant to be a public medical official in an atmosphere of absolute monarchy,
cameralism, and the police state.

Unfortunately, very few of Frank's writings have so far appeared in English
translation. For the reader who is interested in learning more about this important
pioneer, the following may be consulted:

The People's Misery: Mother of Disease. An address delivered in 1790 . . . .
translated from the Latin, with an Introduction by Henry E. Sigerist, *Bull. Hist.
Med.* 9 (1941)81-100.

The Civil Administrator—Most Successful Physician. . . . translated from the
Latin with an Introduction by Jean Captain Sabine, *Bull Hist. Med.* 16 (1944)289-318.

For accounts of Frank's life, see: K. Doll. *Dr. Johann Peter Frank 1745-1821.
Der Begründer der Medizinalpolizei und der Hygiene als Wissenschaften. Ein
Lebensbild.* Karlsruhe, G. Braunsche Hofbuchdruckerei und Verlag, 1909.

Leona Baumgartner and Elizabeth M. Ramsey: Johann Peter Frank and his
"System einer vollständigen medicinischen Polizey," *Ann. Med. Hist. n.s.,* 5 (1933)
525-32; 6 (1934)69-90.

Henry E. Sigerist. *The Great Doctors.* New York, 1933.

MANY YEARS have passed since I reached the summit of the hill which had been assigned to me as the course over which I would run my race. On the other side, perhaps close at hand, perhaps farther down in the valley, the grave awaits me, yet I do not fear it. Meanwhile, I listen thankfully to the voices of my children, and of those whom I regard equally so, my numerous students. They gently insist on the story of my life, and it is important to them; but is it also important to the multitude for whom they intend it? . . . Oh, indeed! Friends at least, and enemies I think, may—each in their fashion—make use of it. The bright spots in my life are for the former; the shadows which it casts upon me for the latter; and the whole for the presumably more objective descendants of all.

Pythagoras says: "A human life resembles Homer's shield, in which, no matter how long one looks at it, one can always discover something new." In such a portrayal should the subject himself direct the pen, or should it be left to others? This is, I admit, a question that is difficult to answer to the satisfaction of all. Nevertheless, if I do not err, a biography is nothing more and nothing less than an account of a journey, which is best described by the traveler, particularly when enough witnesses are still alive to confirm the truth of his tales. Many a person, if he were able to attend his own funeral services and listen to the story of his life, would either steal away fearfully, or at least (if he had laid aside with his mortal frame only the coarser sides of his vanity) would have to redden with shame and cover his face with the shroud. Why, then, leave that which one knows best for others to say (assuming that after one's death there will be something more to say than just: He was born, he lived, and he died)? Therefore, I am writing my own story up to the present. The remainder, presumably very little, may be added after the completion of my arduous journey, if it appears to be worth the trouble, by those who knew me intimately.

I seriously advise those who are to be future citizens of the world to choose for themselves parents who are vigorous, intelligent, and good-natured, whatever their station in life may be. Not only in the case of hunting dogs and horses does it depend on the race whether they will be endowed in greater or lesser degree with those qualities that are necessary for the fulfillment of their destiny. As this counsel is based on experience, there is no reason why I should not take this occasion to speak

had never heard anything more. From all this my father's friend learned that in the strangest way he had discovered my father's mother; only the fear of disappointing himself and the old woman led him to resolve not to speak to his friend about this occurrence until his return. My father did not waste a moment in discovering the truth. All the circumstances coincided precisely. My father's mother, for it was she, had not found any of her relatives at Mannheim or Heilbrunn. All of them had fled because of the war. Bereft of her husband and son, the unfortunate woman had come to the place where she was now found, near the place where for so many years her son had lived without knowing that his mother was so close to him. After the little ready money that she had was gone, the family with whom she now lived had taken pity on her, and she had found a home with them. Now the happy mother embraced her children and grandchildren, and lived to the age of one hundred and five.

I was born at Rotalben on March 19, 1745.[2] Despite his love for his family, my father, who had been raised as an orphan, and had made something of himself only by exerting his energies to the utmost, always retained a harsh streak in his otherwise good-natured character. His fits of rage were often frightening to his children. On one such occasion, when I was nine months old and my mother to quiet my loud cries put me at her breast, he ordered her to leave the room with me immediately! She, however, hoped that she would be able to quiet me. The order was repeated a second time, and when he saw that it was of no avail, he tore me from my mother's breast, and threw me out of the house through the open door. Immediately he realized what he had done, and in despair regretted his rage. On the other hand my mother had the kindest, most gentle character, and in every respect was far above the social station into which she had been born.

One summer day, when I was about four years old, I was sitting alone in front of our house, building a little house out of sand. Suddenly some twenty peasant boys, who were playing there too but probably had not seen me, fell over me. My mother, who thus suddenly lost sight of me, dispersed the boys with her frightened cries. I lay on the ground almost

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[2] Johann Peter Frank must have been one of the last of the children in his family, for as a seven-year-old he already had a married brother.

suffocated and crushed, and from that time on I had frequently recurring attacks of asthma that disappeared completely only when I was eighteen years old. At that time my father dealt in salt. I do not know what pleasant taste I found in it, but I. did consume a considerable quantity of salt every day; and this was probably the cause of the very distressing burning pain on urination to which I was subject for many months. Soon thereafter a large gland in my neck became acutely inflamed and began to suppurate, producing a considerable swelling. Nowhere in the entire district was help to be had. A regimental surgeon from Pirmasens who was consulted, doubted that I would recover, because, as the learned man gave to understand, one of my nerves had burst asunder! On her own responsibility my mother had this swelling opened by a barber who could hardly be induced to perform the operation, and in this way I was once again saved from suffocation. These circumstances, as well as my extreme susceptibility, prevented my father from using me in his hard work as much as he wanted. After he had taken me with him several times in bad weather to Dieuse in Lothringen (Lorraine), and to the fair at Frankfurt, he must have convinced himself that I was not suited for his business, because he said to my mother, after his fashion: The boy is a square peg in a round hole. My mother turned this remark to my advantage, and I was treated with more consideration because of my weakness.

Soon I recovered my strength. With other boys I attended the village school, and although my teacher frequently said that I even knew how to read the Bible, I received many a blow like the others. Once when I was six I came home crying. Questioned as to the reason, I said, sobbing, to my mother: "I am very unhappy to be raised so strictly." To the question, why? I replied: "Because all my school companions are allowed to run about without stockings, while my feet almost suffocate in shoes and stockings!" Aren't the complaints of entire peoples against salutary regulations frequently just as childish?

Finally, when I was seven, my mother sent me to live with Johann Frank, one of my older brothers who was already married, at Eusserthal near Landau. There, for two years, I received instruction appropriate to my age. When I returned to my paternal house, I expressed a desire for further instruction, and asked my mother to allow me to study, without knowing exactly what this meant. At first my father rejected this idea.

But my good mother, who in secret nursed the pious wish to see me dedicated to the church in case I did study, by entreaty obtained permission to send me to the Latin school of the Piarists at Rastadt in Baden. Here, for two years, I was left to my own guidance, which was probably for the best. I learned very little, but at the same time I was not led astray, and lost some of the roughness of my first rustic training. Many things probably contributed to this, among them the frugality with which I was maintained. For my pocket money there had been set aside only one *groschen* a week, which I had to fetch on a certain day from the wife of General von Dreger. Often I was greatly depressed by a debt burden of three or four *groschen* for fruit that I had purchased. Then for the rest of the month I kept away from the spot where my creditor, the woman who sold fruit, was to be found, until finally, red with shame, I was able to settle my debts.

One anecdote I ought not to omit here. At the age of ten, I had a very pleasant soprano voice. The pupils of the Piarists often presented plays in their theater. One time I was given a female part, where my singing of a beautiful aria was received with such loud applause that the reigning Margravine, who was very fond of singing, conceived the idea of sending me to Italy, presumably to have me gelded so that I would retain my soprano voice. However, the aforementioned general assured her that as the son of a well-to-do citizen I had no need to purchase my maintenance so dearly.

Finally, I returned to my home because of an acute quartan fever that had attacked me. At that time there was still considerable prejudice against Peruvian bark, so that when other remedies proved to be of no help, I was told to go to a nearby brook carrying a crayfish in my hand, and to throw it backward into the water. Even now I still take pleasure in the fact that I placed no trust in such a silly business; instead, on returning from the stream I told my mother that in the course of my expedition, the crayfish almost laughed himself to death. The reward for such mockery, however, was that I still had my fever for a long time, and missed a good deal of schooling. Finally my father sent me off to the Jesuit school at Bockenheim (Boucquenom) in nearby Lorraine. My industry grew daily, but only rarely did I reach the top of the class. I had the greatest aversion to learning things by heart, and was therefore never able to recite my lessons properly, no matter how well I under-

stood their content. Because of this I had to suffer more than one humiliation. Here, too, I withstood youthful temptation. As I found great pleasure in music, I began to learn a wind instrument. My good teacher, who regarded me as much too weak for such things, forbade me very strictly to continue with it, and advised me to take up string music. This passion for music has remained with me up to the present, even though I never got very far in putting it into practice.

After three years I was sent to a school in the Margraviate of Baden to learn rhetoric. My teachers soon noticed that I showed considerable interest in this subject, as well as in poetry, and they felt that with my talents I was capable of going on to more advanced studies. After this year of schooling, my mother was advised to send me to France to complete my philosophical studies. As a result, I went to Metz in 1761, and the next year to Pont-a-Mousson, where P. Barlet, a very learned and famous Jesuit, taught physics. The physics museum was so splendidly arranged, and I became so attached to this science, that later in the same year after receiving the doctorate in philosophy, I chose to study medicine because it was affiliated with physics, and to the great sorrow of my pious mother, abandoned all intention of becoming a cleric. My father was likewise very dissatisfied with my intention, and declared that because of the great expense involved, he would be unable to support me. I was disconsolate, and found it impossible to agree to the choice of any other profession as a substitute for my favorite study.

There was, however, still another reason for my state of mind. At Pont-a-Mousson, I had been recommended to M. Pierron, a tradesman, and had been very kindly received in his house. He had a very beautiful and extremely well-educated daughter of my age. Without knowing anything about love, I loved this excellent creature with all my heart. Because of shyness I did not reveal my feelings to her until I saw myself forced to leave her. The first and last time that I was able to speak alone with her, she admitted, with equal shyness, her favorable inclination toward me, adding that if I had not been on the verge of departing from her, she would never have revealed her feelings toward me. I could hardly grasp my good fortune, and at the same time could hardly contain myself for fear that I would have to give it up immediately, when the object of my love and the cause of my despair left me. At least, I called after her, at least may I see you again one day, and if I have been worthy of

against himself in the presence of his auditors. Unfortunately, the victory, which was not a very difficult one, remained on my side. Harrer now lost his temper, resorting to expressions that led me to leave the lectures and never to attend them again. Soon thereafter, my relative pointed out to me in a most serious vein that I had forfeited my happiness, but I did not let myself be talked into apologizing.

In 1765 I moved to Strassburg. Here I attended the lectures of Spielmann,[6] Pfeffinger, and Lobstein.[7] As I knew that he himself had never seen a patient, Spielmann's lectures on pathology according to Gaub made no impression at all on me. Lobstein lectured in most learned fashion on the principles of surgery, but in a tone of voice that put one to sleep. On the other hand, this worthy man gave excellent instruction in anatomy. I became well versed in dissection, and a number of my fellow students soon gained so much confidence in me that they requested me to review physiology with them. I did this gratis, and in teaching them learned much myself. The famous teacher Fried[8] no longer taught obstetrics. Consequently, I chose to study with the accoucheur Weigen, from whom I obtained enough opportunity to train myself in obstetrics. Because of age the experienced Leriche, Surgeon-General of the French Armies, was unable to demonstrate surgical operations as he used to do. I regretted this greatly, but attended the demonstrations on the same subject of his skilled son.

I took an extremely great interest in acquiring practical knowledge. There were two ways to do this; in the civilian hospitals and in the military hospitals. The former had two sections; in one Catholics were treated, in the other Protestants. What medical purpose this served, I do not know. I inquired as to which of the two hospital physicians was considered more skilled. All agreed on the Protestant, and I put myself under his guidance. Numerous other young physicians also accompanied this able physician, Dr. Böhm, when he visited the patients, all too many, whom he treated. In order to obtain still more opportunity for improving myself, I went to the military hospital, and applied to Dr. G., who had often been consulted by the Badenese court. I received permission to accompany this skilled French physician on his rounds in the hospital.

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[6] Jacob Reinhold Spielmann (1722-1783), apothecary and professor of medicine at Strassburg. There is a full figure silhouette of Spielmann in his *Institutiones materiae medi-* cae . . . Strassburg, 1784.

[7] Johann Friedrich Lobstein (1736-1784), professor of anatomy and surgery in Strassburg.

[8] Joh. Jacob Fried (1681-1769).

He visited more than 200 patients there. On his right stood a surgeon; on the left an apothecary; then followed the orderly on duty. The surgeon and the apothecary each kept a list, the former of blood-lettings, clysters, blisters, and so on, the latter of cathartics and other medication. Bed No. 1—Here the doctor glanced to the left and right at the two lists. "Jean," he said, "how are you?" "Very bad, Doctor," was the reply. "Have you been bled?" "Yes, sir!" "Have you taken the purge?" "Yes, sir!" In the meantime the physician felt the man's pulse for a moment with two fingers, and called out in a loud voice: "Bleed him!—Purge him!" While the surgeon and the apothecary were noting these orders as quickly as possible, the doctor was already at the next bed, repeating the same questions and orders. In a half hour we had visited all our patients. I was horrified at this mode of procedure; nevertheless I returned to the hospital for three successive days. However, I found it impossible to witness such a state of affairs any further and renounced such instruction. G. took this lack of confidence on my part with such ill grace that I was accused before the minister of the Badenese court of being remiss Böhm treated me more justly.

In the summer I left Strassburg. The sincerest love for my incomparable Katisch (Katharine) had protected me from all depravity in this seductive place. I lived only for her, and the thought that by applying all my energies to science I could render myself worthy of her and hasten my happiness, enabled me to overcome successfully all difficulties, no matter how great. On the occasion of a repeated visit, she was extremely satisfied with my training, and now I finally obtained from her the promise I had so long desired, that despite all obstacles she wanted to become mine. True love is neither too forward nor compliant. Ours was as pure as the sun.

Now I returned to Heidelberg to submit to the academic examinations. Overkamp, as Dean, received me very coolly. "You are still very young," he said, "to become a doctor." "Unfortunately, I am," was my reply, "but perhaps not unworthy of your attention and good-will!" At that university it is customary to precede the final examination with a *Tentamen* lasting three hours, which is much harder. In this ordeal I was very fortunate because Overkamp who had pressed me hard was again won over completely to my side. Hardly an hour had elapsed when I was told to retire. Fear filled my being at this unusual procedure!

I regarded myself as the most ignorant, the most unfortunate of all mortals. After only a few minutes, however, I was summoned again, and the Dean informed me in the name of the Faculty: because they were so extremely well satisfied with me, I was excused from the remainder of the preliminary examination. The result of the final examination was likewise very favorable for me. Not unjustly, I ascribed my triumph to love, for it had played a most important part in my industry and my rapid progress, something that is rare indeed. In Strassburg, I had already written my inaugural dissertation, *De educatione infantum physica.* I gave it to Gattenhof to judge. He praised the idea, but felt that the execution of it had been less successful. He promised to revise and to shorten it for me, and wrote the fine dissertation, *De cunis infantum,* which appeared under my name, and which I defended publicly on August 26, 1766 before a large audience. In my *Delectus opusculorum medicorum,* published at Padua, I named the true author of this treatise; nevertheless, it was later translated into German, and two years ago also into French, and again ascribed to me, which I did not deserve.

Before I left the university, I was called to Privy Councillor v. Overkamp. "You are very talented and diligent," he said, "and therefore have a right to want to distinguish yourself one day. For this reason pick some subject now on which you would like to work in addition to your professional activities. In three days I expect your answer." I excused myself with my youth, my inadequate knowledge of those fields that needed more cultivation and yet would not exceed my ability. My excuses were rejected, and I was told to return at the appointed time. Great was my embarrassment in the interval! I appeared fearfully before my teacher, and said that I had considered all the branches of [medical] science without finding one that I felt capable of improving. "However," I said, "in the meantime one idea in particular has forced itself upon me. I see that physicians are rarely in a position to remove such causes of disease as either act on people in the mass or are independent of the will of individuals, no matter how careful they may be. Many of these could be removed through governmental measures. Is there a systematically developed science that contains rules according to which such an aim could be achieved?" "We have," replied the professor, "various individual regulations that belong in this field, but no connected scientific system of this kind has yet been developed. Your idea is a happy one. What

would you call your brain child?" "From a medical point of view the object of my investigation would be fixed," I replied, "and since the administration of measures dealing with the health of the public would for the most part have to be left to the police of a country, it seems to me that the name Medical Police would be very appropriate for the subject." My teacher was also in complete accord with this, and once more he urged me very seriously not to abandon my intention.

Now I returned to my parents who had done so much for me. As there was no trained physician to be had, except in the nearby town of Pirmasens, unless one travelled five hours, I soon had a number of patients to treat among the rural population. I had the good fortune to deal successfully with several striking accidents, and for a beginner I did well enough. Yet I could not decide to live alone in a village, deprived of all scientific intercourse. I therefore petitioned the court of the Margrave of Baden-Baden for permission to practise medicine in the Prince's capital city, Rastadt. Presumably because of the aforementioned unfavorable report of the Strassburg military physician G., I received the reply: "... that the request could not be granted as long as I had not practised my profession for some time in a hospital or in a city." I recognized bitterly the meaning of this statement, and looked around for some other place.

At that time my brother Martin Frank lived at Bitsch in Lorraine. He wrote to me that in this small city there was only one physician, a Dr. Landeutte, and he did not know German, so that everyone there wished to have a German doctor settle among them. Immediately I decided to meet this desire. My reception was very polite, but because of my youth I could not expect that the people would immediately have full confidence in me. An old ignorant bath-house attendant already possessed this confidence so completely that the garrison doctor stationed there only rarely had any patients to treat, aside from the military. After several weeks I was summoned to the Lieutenant du Roi, who inquired from whom I had received permission to practise medicine in France. I appealed to my medical diploma, and expressed my amazement that such a question should be put to a graduate physician, when it was thought possible to entrust the health of the citizens to an ignorant bath-house attendant. The Lieutenant du Roi in return referred to an existing royal order, and directed me to the university at Pont-a-Mousson

if I wanted to settle in Lorraine as a physician. Not the examination, but the new expenses connected with it discouraged me; in the meantime I had to accept the situation. I even considered myself fortunate, because in this way I came back sooner to the place toward which my desires were always directed anyway. When I applied to Professor Jadelot, the dean of the Medical Faculty at Pont-a-Mousson, he informed me that I would have to submit to a new examination, and would also have to publish a thesis and defend it publicly. I realized that I would not be able to stay there as long as would be necessary to prepare even a moderately good inaugural dissertation. As I had brought with me a considerable number of copies of the dissertation I had defended in Germany, I conceived the idea of changing the title-page, and having a new one printed here. The Dean agreed to this. I was then examined, and passed to the satisfaction of everyone. At all the street corners a poster written in Latin announced the day when a foreigner would be graduated by the Medical Faculty. At the appointed hour a university proctor appeared in my dwelling bringing me a long black toga, and a black cap shaped like a sugar-loaf, adorned with a silk tassel on top. As I had not ordered a carriage, I refused to go through the streets in such a garb, but all objections were rejected. In this costume, without arousing much interest, I went to the great hall where I was to defend my propositions. Although it was vacation time, I found a large number of people of all kinds collected there. Because I was already a doctor of medicine, two lecterns had been set up; one for the chairman, the elder Professor Jadelot, the other, somewhat lower, for myself. A scholar attacked my propositions in French, and as I was well versed in this language I answered his objections to the satisfaction of all. There was, however, among the so-called corollaries that were appended to my dissertation, one that dealt with small-pox inoculation, and denounced the prohibition of this operation as injudicious. A priest, who was a member of a religious order and also a professor at this university, vigorously attacked this proposition, and declared very impetuously that I "a foreigner was bold indeed to cast aspersions on the conduct of the chief parliament of France, which recently had energetically forbidden smallpox inoculation." Jadelot, who sat behind me, whispered: "This man is dangerous. Be careful!" I realized the critical character of my position, and said to my opponent in his native tongue "that I as a foreigner knew nothing of this new prohibi-

tion, and indeed had not the slightest intention of reproving the actions of such an august body, even though I personally could not approve of them." To the very attentive audience I now admitted that my dissertation had been printed in Germany only seven weeks earlier, and had been given simply a new title-page. I asked that my statement be regarded purely as a medical proposition. "But you, Sir!" I continued, "to whom as a debater victory appears to be very important, would have to be ashamed of your victory if unequal weapons were employed between us. French is not my native tongue; let us therefore use that of scholars, namely, Latin (for I knew that but few French scientists liked to use it), and do me the favor to repeat your objections in that language!" My opponent was extremely perplexed by this turn in the situation, and forced himself to stammer forth a few incorrect Latin words. At this, the audience, which appeared to dislike him anyway, broke out in laughter, and applauded me with a loud Vive l'Allemand!

On October 10, 1766, I was registered as an approved physician of Lorraine,[9] so that there was nothing more to prevent me from practising my profession in this province. I hastened victoriously to the arms of my beloved, and received permission to ask her father for her hand. As the father wanted his only daughter to be happy, he approved her choice. We agreed that until our marriage should take place, she would stay at a convent in Boucquenom and learn something of the German language. Several months later I saw her again, and as she appeared to be making little progress in this difficult tongue, our marriage was not postponed any further, but was celebrated on February 26, 1767. I hardly knew how to describe my joy. Mortals can never achieve anything greater; but, unfortunately as human joys tend to be, it was of very short duration!

Little by little, I found quite a good deal of work in Bitsch and the surrounding districts. As I had no fixed salary, the fees from my practice did not suffice to cover my absolute necessities, and often I had to resort to my mother's kindness. How many young physicians, who lack such sources, and, as I did, marry before they have a secure income, must repent such rashness the rest of their lives!

I had been in Bitsch not quite two years when I decided to return

[9] The phrase used by Frank is: "dem Kollegium der Lothringischen Aerzte einverleibt."

to my native land. I now received permission to settle in Baden, in the Margraviate. Here there was only an elderly physician, an adherent of Stahl's school of thought,[10] who had formerly been personal physician to the Margrave of Baden, but who, several years ago, had retired with a pension to this spot where he was medical officer for a large district and enjoyed the salary attached to the position. I decided to gain the friendship of this experienced physician, Court Councillor Bellon,[11] and to use it for my improvement. However, as he was now much given to drink, and had no desire to see a second physician enter his preserves, my efforts to win his friendship were at first of no avail. After a short time I gained the confidence of the local public, and of the many strangers who visited this famous bath in the summer. A year later, Bellon had become completely senile and was almost useless. In consequence, the court appointed me to carry out all the duties of the district medical officer and gave me 100 florins from Bellon's salary, but with the reservation that when the position became vacant I was to have no prior claim to it (for Dr. Glückherr, a court physician, had his eyes on it).

Both in Bitsch and in Baden I worked with untiring diligence to improve myself. Keeping in mind my teacher's remarks, I had labored valiantly at my work on medical police, and was now resolved to have such a book published in one volume. As a result, I looked around for a publisher, and thought I would make him a rich man with my work. Maklot, a bookseller at Karlsruhe, was to be the recipient of this good fortune. He told me that he first wanted to have an expert opinion on my manuscript. A few weeks later I received my work with the comment that the expert had not approved it. "What? Expert?" I exclaimed, red with shame, and threw the letter from me. After a brief interval, I read the rest of the letter, and discovered that the man who had so disparaged my work was an excellent statesman, Privy Councillor Reinhard of Baden-Durlach. How humiliated by this development was my youthful scientific conceit! This humiliation had an exceedingly great effect on me.

[10] Georg Ernst Stahl (1660-1734), from 1694 to 1716 professor of pathology, physiology, botany and pharmacology at Halle. In 1716, Stahl left Halle for Berlin where he became court physician. Stahl developed the phlogiston theory of combustion, which was not displaced until Lavoisier's discovery of oxygen. He advocated a vitalistic theory of disease, asserting that disease is simply the tendency of the life principle (*anima*) to reëstablish the normal state of the body as rapidly and efficiently as possible.

[11] Georg Martin Bellon, doctor of medicine and philosophy, wrote a book on spas in Baden entitled *Tentamen Physico-Chymico Medicum in Origine Thermarum Badensium.*

Instead of improving my work, I made a second stupid mistake. I took my despised manuscript with both hands, tore it furiously into a thousand pieces, and delivered these to the flames. Would it not have been more intelligent, if I had kept this youthful work, which was probably not quite so bad, to reread it in later years and thus to see how much progress I had made? That is what an excellent Italian sculptor did. Over the door of his house he had set up several mediocre figures, which, in reply to my inquiry, he told me were his own work. When I asked why he had kept them, his answer was that from year to year the wise man judges himself by his own work. In addition, everything that I had previously read, and had abstracted from useful books concerning my subject, was forever lost. These were my reflections when, after calming down, I began my work again. However, I learned so much that I observed more than the Horatian *nonum prematur in annum*, and now spent eleven years in preparing alone the first volume of my Medical Police.

Reader, spare me from saying much about the most terrible fate that now befell me. Eleven months after our marriage, on January 2, 1768, my excellent wife had made me happy with a son, but already on the fourth of that month, she, whom I had so dearly won, was no more! A dreadful childbed fever had attacked her immediately on the first day after her delivery. It was impossible for me to treat her myself. A physician who was summoned from Rastadt bled her twice, because he considered the case one of inflammation of the intestines, whereupon she swooned repeatedly, and finally expired. Before this happened, I was torn from her side, and dragged to the house of my friend. As I had neither eaten nor drunk anything for three days, I was forced to sit at the table next to my friend Court Councillor Braun and to eat something. Hardly had I taken the first bite, when I heard the death-knell. On the spot, I suffered such a severe nose bleed that I was put to bed in an unconscious state. An apothecary who was called as quickly as possible, for lack of a physician, gave me a strong dose of opium, whereupon I fell asleep for thirty-six hours. During this time, my forever unforgettable one was buried.

Still, there remained with me a dear pledge of her love! Only this could reconcile me to live. The wife of a friend of mine, who had likewise given birth a few days previously, took pity on my motherless son,

and suckled the child together with her own. Six months later my mother came to Baden, and urged me to leave my son with her for the first few years. As a very virulent smallpox prevailed in the city, I agreed. During the trip the child began to show signs of impending smallpox, and only a few days after the appearance of the rash, he was snatched from me.

According to the customs then prevailing in Lorraine, because no marriage contract had been drawn up, the fixed property of the deceased, despite the fact that her child had survived her, was inherited neither by myself, nor by my father-in-law, but rather by her maternal family. I had a claim only to the mobile property. As my old father-in-law would have been greatly embarrassed by my claim, I voluntarily ceded it to him, feeling that in this way I was honoring as best I could the memory of his daughter.

For some time I was unable to continue my work. In this condition I received an order to proceed, together with Court Councillor Dr. Bellon and Court Physician Dr. Glückherr of Rastadt, to the nearby town of Gernsbach (in the Badenese County of Eberstein), and to investigate a very dangerous epidemic. After carrying out this mission we went to Rastadt to speak with the personal physicians of the Prince about the epidemic, and then to deliver our joint report to the government. Owing to the illness of the Margrave the physicians were unable to see us before the following day. On this occasion I was able to convince myself that friendship and enmity among men often rest on very shaky grounds. Since I had come to Baden Dr. Bellon had given me various proofs of his hostility, which I was unable to overcome in any way. We had undertaken our small trip without any servants, and had to spend the night at an inn in Rastadt. In taking off his shirt, Bellon who was accustomed to a great deal of personal service, was unable to get out of it because it was securely fastened at the neck. I hastened to help him, and carefully put the feeble old man to bed. For this small favor he now embraced me, admitted that he had been mistaken in me, regretted his actions, and assured me of his inviolable friendship. The next morning he felt homesick, and as the doctors were still unable to meet with us, he hastened home, even though the report on the Gernsbach epidemic had not yet been drafted. I requested him at least to leave behind him in writing his as yet unexpressed opinion. He wrote, and when he had

departed, I read the following: "I the undersigned am of the same opinion as Doctor Frank." After we had presented our report to the government, I received an order to return to Gernsbach, to stay there as long as the epidemic continued and to treat the sick. This I did with much zeal and with such happy success that in six weeks I lost only three of my patients. Prior to my arrival many had died every day from the epidemic. After such a long period of service six Louis d'or were generously given to me as a recompense! Board and lodging I had received free of charge. The disease that I dealt with was a dangerous, so-called putrid or nervous fever with petechial and miliary rash. As the disease began immediately with great debility I applied a stimulatory type of treatment during the first few days. I had been occupied hardly ten days with these patients, when I too was attacked by the disease. As I had been completely well, apart from my emotional conditions, I employed Sydenham's method without losing any time. I took an emetic, went to bed, drank warm, volatile beverages, and, what would now be called good Brunonian treatment, also a bottle of Burgundy. As I was well covered, I soon began to perspire and this continued for forty-eight hours. On the third day I was completely without fever. According to Sydenham's advice I was now supposed to take a purgative, but it appeared to me not to be indicated.

In 1769, Dr. Bellon, the medical officer, died, and his position was now given to Dr. Glückherr, the garrison and court physician. The reigning Margrave appointed me to succeed the latter. However, owing to the fact that an aristocratic lady had very urgently intervened in favor of Dr. Bierenstiel in Rastadt, the Prince divided Glückherr's positions, and gave that clever young doctor the garrison, while I received only 200 florins annual salary. Later I moved to Rastadt. The outlook for science was still very dark there, while in nearby Karlsruhe it flourished. The princely library was rather rich in old books, but for many years nothing had been spent on it, and it had even been closed. P. Lambla, the confessor of the reigning Princess, was in charge of this book collection. At my request I received permission from him to use this treasure. I had to send for the keys every time, but could not get them as often as I would have liked. Nevertheless I did not let myself be put out so easily; never did I spend my free time in a lonelier and quieter place than this court library. The newer writings I had to acquire

out of my own funds, to which my meager salary did not furnish any significant contribution. Between Dr. Bierenstiel and myself prevailed a competition which was very advantageous for both of us, and we exchanged the books that we acquired. As if I had foreseen that I would one day be employed in Italy, I engaged an Italian language teacher, and subscribed to the Florentine newspaper so as to be able to read the writings of Italian physicians. Later this knowledge stood me in good stead. As the two physicians-in-ordinary of the Margrave, Drs. v. Troxelle and Wolff were able to attend only a few patients because of a reported communicable disease at court, most of the patients finally had recourse to me, so that I was extremely busy both in the city and the country. Nevertheless, I frequently consulted with these two older practitioners. During the first years of my employment there, I was called to see a citizen whom Dr. Troxelle had already treated over a long period of time for a generalized dropsy but without success. I did not want to prescribe for the patient without informing his physician, and therefore went to Troxelle who had treated the patient with solvent, mildly purgative and diuretic remedies, in accordance with the then prevalent conceptions of dropsy, and because the patient had a very pallid yellow color. Troxelle advised me to continue the aforementioned medicaments. I replied that this dropsy had been produced by a neglected quartan fever; and that it seemed to me, because this intermittent fever was still active, although much weaker, it was necessary to deal with it first and then with its immediate effect, the dropsy. "With the Peruvian bark that you want to give," said Troxelle, "you will shut up the wolf in the stable, and increase the constipation of the viscera!" "But is this actually present?" I replied. "And if it is present, may it not be like the dropsy, a result of the weakness, or of the fever, and require the same mode of treatment as these?" This opinion was rejected as unorthodox (Troxelle had studied medicine in France). But since I saw that with the previous treatment the patient would soon die, I gave him the Peruvian bark which was feared so much and had the good fortune to restore him completely in fourteen days. This case convinced me that sound reason in no way contradicts experience, which is so highly praised, and that one does not always have to be a graybeard to make use of the former. Soon I was called to a famous ballet master, an Italian named Curioni, who was very popular at the court. He had been attacked by

a very dangerous dysentery. As I found the abdominal pains as well as the urge to stool very severe, and the pulse hard and tense, I bled him five times in three days, and gave him no purgatives, but rather very mild mucous remedies. The doctors and the public criticized my treatment unmercifully, and my growing reputation would have disappeared had the patient done them a favor by dying. Fortunately, however, he recovered. Soon thereafter a report arrived from Strassburg that a number of people there had been attacked by dysentery, but that this illness which had been accompanied by an inflammatory fever had been relieved in most cases by venesection and debilitating remedies. Thus, I was again convinced by experience that if in the case of an imperfect science like medicine we would always leave things as they are, it would never develop its potential possibilities.

As obstetrics had not been learned by any surgeon in this district, much less by midwives, I found myself compelled in serious cases to carry out the deliveries myself. At the request of the Prince, I drafted a plan for the instruction of midwives and surgeons in the lands belonging to Baden-Baden. It was not put into effect, although later it did not escape the attention of the Baden-Durlach government.

In 1770 my relatives and friends urged me to marry again, because they saw that my small competence was being dispersed, owing to lack of attention and because my servant was robbing me. Although I was but little inclined to enter a new union, I followed this advice, and on June 12 married Marianne Wittlinsbach, a daughter of the Chief Clerk of Rastadt. Shortly thereafter I was attacked by a contagious fever. My condition became so bad, that toward the end of the illness I lay in a trance for four hours. Shortly before this illness, I had complained personally to the Minister that my salary was too small. He asked me whether I knew famous old Dr. Wepfer of Switzerland, and I replied that I knew him only by his reputation which was very high. "Now," he said, "I once asked this famous man, why little Switzerland has relatively more distinguished doctors than other bigger countries? To this Wepfer replied: 'Because Switzerland lets 75 out of every 100 young doctors starve to death, with the result that the remaining 25 become rather good physicians.'" I requested the Minister not to undertake such experiments on me, and was of the opinion that many a useful talent had been stifled in this way. After my recovery from the acute illness, my salary was increased by 100 florins.

In the meantime my eighty-year-old father died. Several years previously it had been my good fortune to save his life which was endangered by a strangulated inguinal hernia. On this occasion he had told me, in his fashion, he was now convinced that there was something to this profession that I had studied. He had received the news of my first wife's death with dismay. "That I, an old man who is no longer of any use to anyone, should outlive such a beautiful, fine young woman!" he had cried out. In his will he ordered that I should participate in the property that he bequeathed to his children only if the sum that I had cost him was less than that which could be inherited by each of his six other children. According to the laws of the country, I could have opposed this arrangement. However, I knew that my father was just. I respected his wish, and stipulated only that my brothers and sisters divide the inheritance without quarreling. Not long after this loss followed the death of my excellent mother, filling my soul with the bitterest grief.

In 1771 the health of the reigning Margrave, August, the last Prince of the Baden-Baden line, became shaky, and he began to show signs of dropsy. At a time when the disease was already quite advanced, his personal physicians were ordered by the Minister to consult me too. Although they were quite friendly with me, they refused, for some unknown political reasons, to obey this order. Finally my presence was urgently requested. The doctors had to obey, but I was not permitted to see the sick Prince on the ground that he would consider his malady more serious and would become frightened. The same pretext was also alleged when Professor Strack, who had been summoned from Mainz, was refused admission to the Prince. This famous physician was on the point of returning to Mainz because of this insult when the reigning Princess ordered that we be admitted to her sick husband even without the cooperation of the attending physicians. The Margrave received us very favorably, and reproved me that I had not visited him before during his illness. We found that the disease was far advanced, and no hope remained for recovery. Nevertheless it was important to prolong the life of the Prince as long as possible. Despite all his suffering, the patient remained quite cheerful. As we left him, I heard him say laughingly to one of his intimate friends: "Now I would like to hear what these five criminal judges will decree for me!" Diuretics had little effect. The two physicians-in-ordinary alternated in maintaining the nocturnal vigil,

and one night when Dr. Wolff was with him the patient urinated more than usual. Thereupon the Margrave requested this younger physician not to say anything about this occurrence to his elder colleague, Dr. Troxelle, because he would be vexed that the Prince had urinated more for the one doctor than for the other.

The most famous physicians of Germany and France were consulted, and in addition to Strack, Doctors Ehrmann and Böhm of Strassburg, as well as Dr. Cohausen of Koblenz and Dr. Glückherr of Baden were summoned. Although Cohausen was already over eighty, he was still in full possession of his mental faculties and remembered everything that he had ever read, or had observed himself. Ehrmann[12] was the leading practitioner of Strassburg. These two old doctors, as well as Strack of Mainz, honored me with their friendship, and often conversed with me for hours on scientific subjects. As much as I could, I took advantage of the society of such experienced men. We dined together daily, and one day we also were on the verge of dying together had I not prevented the misfortune in time. We were served a silver plate full of the finest trout. They were in a grass green sauce, which some of us believed was colored by chervil, and others by some other fresh vegetable. I recalled, however, that these fish had been boiled in vinegar the previous evening, and having lain on this plate all night were probably saturated with verdigris. Upon closer investigation my presentiment was completely confirmed, and the highly esteemed gentlemen had to forego a fine dish. On one occasion I found myself alone with the sick Prince. He commanded me to tell him honestly and conscientiously whether there was still any hope for his recovery. Knowing his manly character, I had no scruple in admitting that there was no longer any hope, and that his end was close at hand. Thankfully he pressed my hand. "Il le faut une fois," he said, and from this moment on he seemed to face his fate with equanimity. A few weeks later, on October 21, 1771, the sick Prince died.[13] With tears in my eyes, I autopsied his body, and found an almost complete ossification of the aorta and its main branches up to their division into the femoral arteries. I reported this to the famous Tissot, whom I was to succeed fifteen years later at Pavia. I wrote to him that not only

[12] Johann Christian Ehrmann (1710-1797). Since 1749 he had been a physician to the workhouse at Strassburg.

[13] With the extinction of the Baden-Baden line, the Margraviate of Baden-Baden was inherited by the Baden-Durlach line residing at Karlsruhe.

the brother of the Prince, who was 54 years old, but also his heir, who was only nine years old, had suffered from a similar ossification of the large vessels. I was more than a little surprised to read in Tissot's reply that if the Margrave had been correctly treated from the beginning, he would have been able to live for a long time with this ossification.

On December 23, 1771, my wife gave birth to Joseph, my eldest son.

On April 1, 1772 all the employees and servants of the deceased Margrave, with the exception of four or five officials, were pensioned off. I retained the status of a Court Physician and received an annual pension of 75 gulden. After a short time I received another appointment, this time as Inspector of Midwives in a section of Baden. This position I could not have kept very long, while simultaneously carrying on a medical practice. However, on his return to Koblenz, the old Privy Councillor and Physician-in-ordinary Cohausen had recommended me warmly to the Prince-Bishop of Speyer, Count of Limburg Styrum, whom he had known for a long time. As a result I was soon offered a position as municipal and district medical officer at Bruchsal with a salary of 400 florins and the status of Court Councillor. I accepted this call, and left my native land, to which I would so gladly have devoted my life.

In Bruchsal it was my duty to attend gratis the garrison hospital, an asylum for infirm old people at Altenbürg, a half hour's distance from the city, the large pentitentiary, the prisons and all the sick poor. In addition the medical district outside the city included 36 localities. I was happy to have a position that offered me so much opportunity for the exercise of my profession, and which long before had also been filled by my unforgettable teacher, Professor Gattenhof. I had been at Bruchsal hardly a year and a half when I received an offer, in private, of a post as district medical officer at Bretten in the Palatinate, and as physician in the nearby spa at Zeisenhausen with a salary of 800 florins. The Prince of Speyer learned of this negotiation which was so advantageous for me, wrote to the Elector of the Palatinate to interrupt it, and increased my salary by 200 florins annually. A few months later, Dr. Brodbeck, Privy Councillor and First Physician-in-ordinary to the Prince of Speyer, a very skilled man, left his position, and the Prince-Bishop offered it to me. As I had now reached a point where I could best increase and improve my knowledge by daily experience at the bedside, I told the Prince that I could not accept this position unless I were permitted also to continue to

serve the local public. He agreed to this, and I now received a salary of 800 florins as well as free lodging, free board, 264 bushels of grain, and two tuns of wine. Simultaneously I also became physician to the local saltworks.

On January 6, 1774 my second son, Franz, was born. When the Prince-Bishop had appointed me his personal physician, he had left me to determine my successor in the position at Bruchsal, so my friend Dr. Bierenstiel obtained this post.

As the principality of Speyer had only one trained midwife at Bruchsal, the Prince-Bishop was much concerned about improving the state of midwifery in the entire country. To this end I drafted a plan, and as teacher of midwifery carried it out for more than nine years with the happiest results. Prior to the establishment of this school, and even during the first years of its existence one woman out of every 85 who were pregnant died in labor, or lying-in; after most of the midwives had been trained only one out of 125 died.

Not even a year had passed since I had become physician to the Prince, when he revealed to me that for several months already he had been afflicted with a hydrops of the tunica vaginalis of one testicle, or with what is incorrectly known as a hydrocele. As I expressed no hope for the cure of this condition with internal remedies, and did not wish to propose a so-called radical cure because of the extreme irritability of the Prince who was sickly anyway, advising instead that the fluid be tapped simply as a palliative measure, the patient against my advice wanted to submit to surgery. I requested a consultation with well-known physicians. They approved the operation, which was performed soon thereafter by the Palatine Surgeon-in-ordinary Winter. As the otherwise healthy testicle was covered with small vesicles, the patient, despite my open protests, ordered that it be removed, and the surgeon had to obey. Recovery was accompanied by serious complications. Hardly had the patient begun to recuperate when there appeared on the closed scar a secretion of watery matter, whereupon the Prince's strength visibly increased. Nevertheless he wanted to have this secretion suppressed, and as I did not wish to do this, because I knew that the Prince had formerly been much afflicted with herpes, and always suffered greatly with nerve ailments upon the disappearance of this condition, he employed a miserable village barber who stopped the secretion in a few days by means of astringents. This

was soon followed by an inflammation of the liver, and when I had cured this, the patient, who had recently exposed himself to various causes of disease, suffered a very frightening mental derangement. In one attack he was about to throw himself out of a high window when I pulled him back, thus endangering my own life. After several occurrences of this kind, the cathedral chapter at Speyer felt it necessary to send a deputation to Bruchsal to request a statement from me on the condition of the Prince-Bishop. In my statement I said that the patient was mentally deranged, but that this was a consequence of preceding diseases and disorders in his mode of life rather than a primary mania and, I flattered myself, could be cured. After several months had elapsed, the successful outcome showed that my prognosis regarding this illness, which finally disappeared completely, had been based on good reasons. I was now appointed Princely Privy Councillor, and my salary was raised by 200 florins.

Soon thereafter the Prince-Bishop founded a small hospital in Bruchsal, and placed it under the direction of the Brothers of Mercy. I was made medical director of this hospital, as well as of a similar one at Deidesheim on the left bank of the Rhine, and received an increase of 200 gulden. Later I was appointed a member of a Princely Commission set up to advance the interests of pious foundations.

As a well-equipped hospital was present at Bruchsal, to which was attached an excellent surgeon in the person of B. Joachim Wrabecz, I took advantage of this circumstance to propose to the Prince that anatomical and surgical lectures be given in this hospital for the benefit of the surgeons. My plan was approved, and Wrabecz took over this task with the best results. I participated myself, trained the students in the art of dissection, and together with the aforementioned teachers laid the foundation for a pathological museum at Bruchsal. As lectures on physiology seemed to promise considerable benefits for our young surgeons, I held these daily at a specified time for more than seven years. During the summer, I went with my auditors once or twice almost every week to botanize in the neighborhood. Finally, the Prince-Bishop gave me a garden in which I raised plants employed for medicinal purposes as well as others, and for three years I instructed young surgeons in the principles of botany.

Since coming to Bruchsal, I had made diligent use of the not inconsiderable library that had been purchased by Cardinal Prince von Hutten from the famous Pistorius in Würzburg, and which was now

kept in the Seminary. Although very few or no medical works were present in this library, yet in the many classical works and in other works concerned chiefly with history and legislation, especially in an excellent collection of polemic treatises on various subjects, I found a veritable treasure for my Medical Police. Such free time as remained from my very extensive practice and my studies, I spent in the company of my good sister (Maria Magdalena Frank, wife of the Princely Governmental-Secretary Lippert), and of my two unforgettable friends, the present Privy Councillor Oehl, and Court Councillor Niesen (author of Algebra for the Seeing and the Blind), whom death has since taken from me, and his excellent wife.

In 1776, I had the following little book printed anonymously by Schwan at Mannheim:

1. *Sendschreiben eines Rheinischen Arztes über einige von dem Kollegium der Aerzte zu Münster aufgestellte Grundsätze.*

Soon thereafter, I followed this with another written in Latin, namely:

2. *Joannis Petri Frank,* M.D. Consiliarii Aulici ac Archiatri Spirensis, *Epistola invitatoria ad Eruditos, de communicandis, quae ad Politiam medicam spectant, Principum ac Legislatorum Decretis.* Mannhemii apud Schwan. 1776. 8vo.

After the appearance of this publication, I read a review of it in a learned journal. The reviewer praised the plan that I had announced for my Medical Police, but he doubted whether the author would be equal to the task, especially as he lived in Bruchsal, and had no access to a public library like that at Göttingen. In time this objection was also to be removed. On the whole, I received few contributions from foreign physicians. Court Councillor Gruner of Jena and Professor Platz of Leipzig sent me important works.

Epidemics prevailing among cattle, and soon thereafter among horses, gave me occasion to carry out several pathological dissections on these animals. The prejudice against this, however, may be seen from the following circumstance. A white mare had died while foaling, and was to be buried in a nearby wood by the knacker. When I wanted to dissect the animal and asked for a larger knife from the knacker, he requested me with the best of intentions "not to damage my honor in this way!" A similar prejudice would almost have ruined the only trained midwife in Bruchsal. Out of pity for a poor family, she had agreed to

save their only possession, a cow that was unable to give birth to her calf, by delivering the animal. As soon as this occurrence became known in the city, almost no woman wanted to employ this midwife. I induced the Prince to give her a reward for her good deed.

In 1779 I issued the first volume of the following work:

3. Johann Peter Frank, M.D. Privy Councillor and Personal Physician to His Highness the Prince of Speyer. *System einer vollständigen medizinischen Polizey.* I. Band. Mannheim at Schwan's. 1779. 8vo.

As this work had no small influence on my fate, I do not feel it necessary to hold back certain circumstances that relate to it. When the manuscript of this first volume was completed, I requested the Prince-Bishop of Speyer, who had no high opinion of learned works, to appoint someone to censor it for me. He chose the Clerical Privy Councillor Schmidt, General Vicar of the Bishopric of Speyer (at present suffragan bishop), a very learned, excellent man, who for many years while still a Jesuit, taught Canon Law at Heidelberg. After carefully reading my manuscript, he certified in writing "that it contains nothing contrary to morality, religion or the political order." At the same time this worldly-wise man predicted many of the effects that my work would produce, but these were unable to frighten me. I now gave my manuscript to the press. Hardly had the last page been printed, when a learned journal at Frankfurt announced it in such a way that I knew very well the reviewer had read only the running heads of my book. Immediately a hullabaloo developed. The publisher wrote to me that by all indications the work would be prohibited as soon as it appeared, and that in certain places it was being subjected to careful official scrutiny. I knew of no crime that I had committed; I was covered by the censor's opinion; and therefore awaited the outcome calmly. A year elapsed before I became aware that this book had made a favorable impression on someone other than its author. At that time, however, the Prince-Bishop reproached me in such fashion that I was led to request my release from the service of Speyer. This was not granted. I learned that the criticism stemmed from a foreign source. The Bishop, whose life I had saved three times, assured me of his satisfaction with me, and I remained in my post, on the condition that I would be able to continue my work.

The first edition was exhausted within the year of publication, and as I had not found anything essential that had to be changed or added,

this volume was provided with a second preface and simply reprinted.

In the meantime I sent a small article to Dr. Joh. Christian Ferd. Scherf's *Archiv der medizinischen Polizey und der gemeinnützigen Arzneykunde.* It was entitled:

4. *Etwas über die Zwistigkeiten der Aerzte und ihre Ursachen.* This paper was published in the first volume (p. 133) of that useful publication. The same volume (p. 151) also contains:

5. *Das Hochfürstlich Speyersche Mandat zur Verhütung der Hundswuth,* Bruchsal, 1779. (I was the author of the medical section.)

A short description of an animal monster that I wrote is to be found in Reinhard's *Medizinisches Wochenblatt für Aerzte, Wundärzte, und Apotheker,* Volume 1, 1780, p. 221.

In 1780 I published:

6. *System der medizinischen Polizey,* II. Band. Mannheim, 1780. 8vo.

The widow of the reigning Margrave of Baden-Baden, née Duchess von Aremberg, resided in Baden. During a visit which she made to the Prince-Bishop of Speyer to attend to various matters, she requested him to permit me to attend her in a professional capacity, and whenever she was seriously ill to visit her as often as I believed to be necessary and the health of the Prince would permit it. As the distance from Baden was no more than six miles, and the Bishop (who had another personal physician, Dr. Roussi) wanted to oblige this Princess, he not only permitted me to take a position as her personal physician, but even commanded me in her presence to respond to her every beck and call. Hardly had the Princess departed, when the Bishop reproached me bitterly for having assumed this obligation. I assured him that I had accepted this position only at his command, and was also ready at his command to repudiate it. Furthermore, I assured him that in case he himself should fall ill, I would never leave him for anyone else. The Bishop forbade me to mention this occurrence to the Margravine. A half year later the Princess wrote to me, and requested my attendance. The Bishop was in good health, and I asked him whether I should accede to this request or refuse it. He told me to obey. I promised to be with him again in three days. Just as I was about to depart, the Prince sent his confidant to inform me that I could attend the Margravine this one time, but not thereafter and for this he would promise me an annual increase of 300 florins. I replied

TABLE 3

Agglutination titres S. dysenteriae and homologous E. coli in patients' serum

PATIENT	STRAIN E. COLI	TITRE FOR S. DYSENTERIAE	TITRE FOR HOMOLOGOUS E. COLI	SERUM ABSORBED WITH HOMOLOGOUS E. COLI		SERUM ABSORBED WITH S. DYSENTERIAE		
				Final titre E. coli	Final titre S. dysenterino	Absorbing organism	Final titre for corresponding S. dysenterino	Final titre for E. coli
MI	II0	E. Led 1 + 1/640 Y. Led 1 + 1/640 KK 4 + 1/320	1 + 1/640	0	E. Led 0 Y. Led 1 + 1/320 KK 1 + 1/320	E. Led Y. Led KK	0 0 0	0 4 + 1/640 4 + 1/640
VC	II3	E. Led 1 + 1/640 PB24 1 + 1/160	1 + 1/320	0	E. Led 0 PB24 0			
S	H6	E. Led 1 + 1/640 PB24 4 + 1/160 KK 1 + 1/160 Y. Led 1 + 1/640	4 + 1/640	0	E. Led 4 + 1/40 PB24 4 + 1/160 KK 3 + 1/160 Y. Led 1 + 1/640	E. Led PB24 KK Y. Led	0 1 + 1/80 1 + 1/160 1 + 1/320	0 4 + 1/320 4 + 1/640 4 + 1/640
G	NII3	E. Led 1 + 1/160 PB24 1 + 1/160 KK 1 + 1/160 Y. Led 1 + 1/160	1 + 1/320	0	E. Led 1 + 1/80 PB24 1 + 1/160 KK 1 + 1/160 Y. Led 1 + 1/160	E. Led PB24 KK Y. Led	0 0 0 0	1 + 1/320 1 + 1/320 1 + 1/320 1 + 1/320
S	NII	E. Led 1 + 1/640 KK 4 + 1/640 Y. Led 4 + 1/160	1 + 1/320	0	E. Led 1 + 1/320 KK 0 Y. Led 0	E. Led KK Y. Led	0 0 0	1 + 1/320 0 1 + 1/160
W	NII7	E. Led 1 + 1/320 PB24 1 + 1/160 KK 4 + 1/80 Y Led 4 + 1/160	0	0	E. Led 1 + 1/320 PB24 1 + 1/80 KK 0 Y. Led 1 + 1/160	Y. Led	0	

W	H7	E. Led 1 + 1/320 PB24 1 + 1/160 KK 4 + 1/80 Y. Led 4 + 1/160	0	0	E. Led 1 + 1/320 PB24 1 + 1/80 KK 4 + 1/80 Y. Led 1 + 1/160	KK	0	0
T	H1	E. Led 1 + 1/160 KK 4 + 1/160 Y. Led 1 + 1/160	1 + 1/160	0	E. Led 0 KK 1 + 1/100 Y. Led 1 + 1/80	E. Led KK Y. Led	0 0 0	0 1 + 1/320 1 + 1/320
A	H6	E. Led 1 + 1/80 PB24 1 + 1/80 KK 1 + 1/160 Y. Led 1 + 1/160	2 + 1/40	0	E. Led 3 + 1/20 PB24 2 + 1/20 KK 2 + 1/40 Y. Led 1 + 1/80	E. Led PB24 KK Y. Led	0 0 0 0	1 + 1/40 1 + 1/40 1 + 1/40 1 + 1/40
W	H12	E. Led 4 + 1/160 KK 4 + 1/160 Y. Led 2 + 1/160	0	0	E. Led 0 KK 4 + 1/80 Y. Led 4 + 1/160	E. Led	0	
L	H2	E. Led 4 + 1/160 PB24 1 + 1/160 KK 1 + 1/160 Y. Led 1 + 1/320	1 + 1/320	0	E. Led 0 PB24 1 + 1/80 KK 1 + 1/160 Y. Led 1 + 1/160	E. Led PB24 KK Y. Led	0 0 0 0	0 4 + 1/320 1 + 1/320 4 + 1/320
L	H1	E. Led 1 + 1/160	0	0	E. Led 0			
G	H2	E. Led 1 + 1/160	0	0	E. Led 0			

both the homologous dysentery agglutinins and the heterologous coli agglutinins. Absorption of the sera by the individual *E. coli* strains removed the corresponding coli agglutinins.

Unabsorbed PB24 serum did not agglutinate any of the *E. coli* strains to full titre (table 4). One strain, S-NH, reached half titre, and two, W-NH and W-H7, agglutinated at one-eighth titre. All the remaining strains agglutinated in serum dilutions varying from 1/160 to 1/1280.

TABLE 4

Agglutinin titres—E. coli vs. PB24 serum

PB24 SERUM UNABSORBED		PB24 AB-SORBED WITH PB24	PB24 SERUM ABSORBED WITH E. COLI VS. E. COLI		PB24 SERUM ABSORBED WITH E. COLI VS. PB24	
			Absorbing organism	Final titre	Absorbing organism	Final titre
PB24	40,960	20				
MI-H6	2,560	20	MI-H6	20	MI-H6	40,960
VC-H3	160	20	VC-H3	20	VC-H3	40,960
S-H6	320	20	S-H6	20	S-H6	40,960
G-NH3	1,280	20	G-NH3	20	G-NH3	10,240
S-NH	20,480	20	S-NH	20	S-NH	5,120
W-NH	5,120	20	W-NH	20	W-NH	40,960
W-H7	5,120	20	W-H7	20	W-H7	10,240
T-H1	1,280	20	T-H1	20	T-H1	20,480
A-H6	1,280	20	A-H6	20	A-H6	10,240
W-H12	160	20	W-H12	20	W-H12	40,960
L-H2	320	20	L-H2	20	L-H2	20,480
L-H1	160	20	L-H1	20	L-H1	40,960
G-H2	320	20	G-H2	20	G-H2	10,240

Absorption with the individual coli strains variously affected the titre for the homologous dysentery antigen. Strain S-NH, which agglutinated to highest titre in the unabsorbed serum, proved to be the most active absorber of the group, reducing the titre for PB24 to 5,120. W-H7, which agglutinated to an eighth titre, reduced the homologous agglutinins to 10,240. The three strains, G-NH3, T-H1, and A-H6, likewise showed active absorption. They reduced the titre for the homologous PB24 antigen to 10,240, 20,480, and 10,240 respectively. L-H2 and G-H2, although agglutinating only to 1/320, reduced the titre

for the homologous dysentery antigen to 20,480 and 10,240 respectively.

The unabsorbed Karim Kahn serum likewise failed to agglutinate any of the *E. coli* strains to full titre (table 5). Only two, W-H7 and L-H1, reached one-quarter titre. The remainder agglutinated in dilutions varying from 1/320 to 1/5120.

Eight of the *E. coli* strains, when used to absorb the Karim Kahn serum, reduced the titre of this serum for its homologous

TABLE 5

Agglutinin titres—E. coli vs. Karim Kahn serum

KK SERUM UNABSORBED		KK SERUM ABSORBED WITH KK	KK SERUM ABSORBED WITH E. COLI VS. E. COLI		KK SERUM ABSORBED WITH E. COLI VS. KK	
			Absorbing organism	Final titre	Absorbing organism	Final titre
KK	40,960	20				
MI-H6	320	20	MI-H6	20	MI-H6	40,960
VC-H3	640	20	VC-H3	20	VC-H3	40,960
S-H6	640	20	S-H6	20	S-H6	20,480
G-NH3	640	20	G-NH3	20	G-NH3	40,960
S-NH	5,120	20	S-NH	20	S-NH	20
W-NH	2,560	20	W-NH	20	W-NH	5,120
W-H7	10,240	20	W-H7	20	W-H7	5,120
T-H1	1,280	20	T-H1	20	T-H1	20,480
A-H6	2,560	20	A-H6	20	A-H6	10,240
W-H12	640	20	W-H12	20	W-H12	40,960
L-H2	640	20	L-H2	20	L-H2	40,960
L-H1	10,240	20	L-H1	20	L-H1	10,240
G-H2	320	20	G-H2	20	G-H2	10,240

antigen to fifty per cent or less. S-NH, although agglutinating only to 5120, completely absorbed the Karim Kahn agglunitins. G-H2, which agglutinated only to 1/320 in the unabsorbed serum, reduced the titre for the homologous antigen to twenty-five per cent.

The unabsorbed Y Ledingham serum agglutinated one of the *E. coli* strains, W-H7, to full titre; a second, S-NH, to half titre; and a third, W-NH, to one-quarter titre. The remaining ten strains agglutinated at dilutions ranging from 1/160 to 1/2560 (table 6).

Nine of the *E. coli* strains, when used to absorb the Y Ledingham serum, reduced the titre for the homologous dysentery antigen to fifty per cent or less. The completeness of absorption, however, did not parallel exactly the agglutinability of the coli strains in the unabsorbed serum. W-H7, which agglutinated to full titre, reduced the homologous dysentery agglutinins to fifty per cent. S-NH agglutinated at half titre and reduced the homologous agglutinins to 1/1280. W-NH agglutinated at

TABLE 6

Agglutination titres—E. coli vs. Y Ledingham serum

Y LED SERUM UNABSORBED		Y LED SERUM ABSORBED WITH Y LED	Y LED SERUM ABSORBED WITH E. COLI VS. E. COLI		Y LED SERUM ABSORBED WITH E. COLI VS. Y LED	
			Absorbing organism	Final titre	Absorbing organism	Final titer
Y Led	20,480	20				
MI-H6	1,280	20	MI-H6	20	MI-H6	10,240
VC-H3	160	20	VC-H3	20	VC-H3	20,480
S-H6	640	20	S-H6	20	S-H6	10,240
G-NH3	640	20	G-NH3	20	G-NH3	10,240
S-NH	10,240	20	S-NH	20	S-NH	1,280
W-NH	5,120	20	W-NH	20	W-NH	10,240
W-H7	20,480	20	W-H7	20	W-H7	10,240
T-H1	1,280	20	T-H1	20	T-H1	20,480
A-H6	2,560	20	A-H6	20	A-H6	10,240
W-H12	640	20	W-H12	20	W-H12	20,480
L-H2	640	20	L-H2	20	L-H2	5,120
L-H1	160	20	L-H1	20	L-H1	20,480
G-H2	320	20	G-H2	20	G-H2	10,240

twenty-five per cent titre and reduced the dysentery agglutinins to fifty per cent.

Four strains, which agglutinated in relatively low dilutions, 1/320 to 1/640, proved to be active absorbers of Y Ledingham agglutinins, reducing the titre of the serum in these instances to fifty per cent and in one to twenty-five per cent.

The results obtained with E. Ledingham (Sonne) serum were striking (table 7). Six of the coli strains agglutinated at full titre, one at half titre, and two at one-quarter titre. One reached one-eighth titre. The remaining three failed to agglutinate in dilutions above 1/320.

The six strains of *E. coli*, which agglutinated at full titre in the E. Ledingham serum, completely absorbed the dysentery agglutinins. The two strains A-H6 and W-H12, which agglutinated at one-half and one-quarter titre respectively, likewise completely absorbed the E. Ledingham agglutinins. G-NH3 agglutinated to one-quarter titre and reduced the dysentery agglutinins to one-eighth. W-NH7 and W-H7, which agglutinated only to 1/320, reduced the homologous agglutinins in the serum to fifty per cent. S-NH agglutinated at 1/320 and reduced

TABLE 7

Agglutination titres—E. coli vs. E. Ledingham serum

E. LED SERUM UNABSORBED		E. LED SERUM ABSORBED WITH E. LED	E. LED SERUM ABSORBED WITH E. COLI VS. E. COLI		E. LED SERUM ABSORBED WITH E. COLI VS. E. LED	
			Absorbing organism	Final titre	Absorbing organism	Final titre
E. Led	40,960	20				
MI-H6	40,960	20	MI-H6	20	MI-H6	20
VC-H3	40,960	20	VC-H3	20	VC-H3	20
S-H6	40,960	20	S-H6	20	S-H6	20
G-NH3	10,240	20	G-NH3	20	G-NH3	5,120
S-NH	320	20	S-NH	20	S-NH	10,240
W-NH7	320	20	W-NH7	20	W-NH7	20,480
W-H7	320	20	W-H7	20	W-H7	20,480
T-H1	40,960	20	T-H1	20	T-H1	20
A-H6	20,480	20	A-H6	20	A-H6	20
W-H12	10,240	20	W-H12	20	W-H12	20
L-H2	40,960	20	L-H2	20	L-H2	20
L-H1	5,120	20	L-H1	20	L-H1	5,120
G-H2	40,960	20	G-H2	20	G-H2	20

the agglutinins to twenty-five per cent. L-H2 agglutinated to 1/5120 and reduced the serum titre after absorption to the same figure.

Findings with monovalent anti-coli rabbit sera

A monovalent rabbit serum was prepared against each of the thirteen strains of *Escherichia coli*. These sera ranged in titre from 10,240 to 40,960.

The agglutinability of the four strains of *S. dysenteriae* was studied in these sera unabsorbed, after absorption by the homolo-

gous *E. coli*, and after absorption by the heterologous *S. dysenteriae*. Absorption of each serum by its homologous antigen removed both the homologous coli agglutinins and the heterologous dysentery agglutinins. Absorption of the sera by the individual strains of *S. dysenteriae* removed the corresponding dysentery agglutinins.

The thirteen sera fall into two groups on the basis of the behavior of the E. Ledingham dysentery bacillus. The first group of five, with the exception of one, W-NH, fail to reveal any antigenic relationship between the homologous *E. coli* and the E. Ledingham Sonne. The second group of eight sera, on the other hand, afford evidence of close relationship between the homologous antigens and the heterologous E. Ledingham.

The five sera of group one (table 8) failed to agglutinate E. Ledingham above 1/640. In one instance, W-NH, absorption of the serum with this strain of *S. dysenteriae* reduced the titre for the homologous *E. coli* fifty per cent. The other sera were not affected.

Varying reactions were obtained with the Flexner strains. The PB24 Flexner agglutinated to full titre in unabsorbed S-NH serum. Absorption of the serum with PB24 completely removed the homologous coli agglutinins. The Y Ledingham and Karim Kahn Flexners, agglutinated at one-half and one-eighth titre respectively. Each completely absorbed the homologous coli agglutinins.

W-NH serum, titre 40,960, agglutinated Karim Kahn to one-quarter titre. Absorption with this organism reduced the homologous coli agglutinins to 640. Y Ledingham, which agglutinated at one-eighth titre, did not absorb the homologous agglutinins. Conversely, E. Ledingham and PB24, agglutinating at 1/640, reduced the serum titre for the homologous coli to fifty per cent.

G-NH3 serum agglutinated PB24 at 1280. Absorption with this strain of Flexner reduced the titre for the homologous antigen to fifty per cent. Two of the other dysentery antigens agglutinated at low titre but failed to absorb.

W-H7 serum agglutinated the three Flexner strains at one-half,

one-quarter and one-eighth titre respectively. Absorption with these organisms reduced the homologous coli agglutinins to one-

TABLE 8

Agglutination titres—S. dysenteriae vs. E. coli sera

E. COLI SERUM	TITRES UNABSORBED SERUM		SERUM ABSORBED WITH S. DYSENTERIAE VS. S. DYSENTERIAE		SERUM ABSORBED WITH S. DYSENTERIAE VS. HOMOLOGOUS E. COLI	
			Absorbing organism	Final titre corresponding organism	Absorbing organism	Final titre for homologous E. coli
G-NH3....	G-NH3	20,480				
	E. Led	640	E. Ledingham	20	E. Ledingham	20,480
	PB24	1,280	PB24	20	PB24	10,240
	KK	0	Karim Kahn		Karim Kahn	
	Y Led	640	Y Ledingham	20	Y Ledingham	20,480
S-NH	S-NH	10,240				
	E. Led	320	E. Ledingham	20	E. Ledingham	10,240
	PB24	10,240	PB24	20	PB24	20
	KK	1,280	Karim Kahn	20	Karim Kahn	20
	Y Led	5,120	Y Ledingham	20	Y Ledingham	20
W-H7	W-H7	40,960				
	E. Led	640	E. Ledingham	20	E. Ledingham	40,960
	PB24	5,120	PB24	20	PB24	10,240
	KK	10,240	Karim Kahn	20	Karim Kahn	5,120
	Y Led	20,480	Y Ledingham	20	Y Ledingham	20,480
W-NH	W-NH	40,960				
	E. Led	640	E. Ledingham	20	E. Ledingham	20,480
	PB24	640	PB24	20	PB24	20,480
	KK	10,240	Karim Kahn	20	Karim Kahn	640
	Y Led	5,120	Y Ledingham	20	Y Ledingham	40,960
L-H1 .	L-H1	40,960				
	E. Led	80	E. Ledingham	20	E. Ledingham	40,960
	PB24	160	PB24	20	PB24	40,960
	KK	5,120	Karim Kahn	20	Karim Kahn	5,120
	Y Led	160	Y Ledingham	20	Y Ledingham	40,960

half, one-quarter and one-eighth titre. Karim Kahn, which agglutinated at one-quarter titre, proved to be the most active absorber.

The L-H1 serum reacted significantly with only one of the Flexner antigens. Karim Kahn agglutinated at one-eighth titre, and when used to absorb the serum reduced the titre for the homologous *E. coli* to a like level.

The remaining eight sera reveal a close antigenic relationship between the homologous *E. coli* and the E. Ledingham (Sonne) bacillus. There is a less close relationship with the three Flexner strains (table 9). Each strain of *E. coli* was hemolytic and each produced a serum with titre of 40,960.

Six of these coli sera agglutinated E. Ledingham to full titre. Absorption of these six sera by E. Ledingham completely removed the homologous *E. coli* agglutinins. The A-H6 and L-H2 sera agglutinated E. Ledingham to one-half titre. This organism completely absorbed the homologous agglutinins from both sera.

The Flexner organisms were not strongly agglutinated by these coli anti-sera. In one instance the titre reached 5,120, in three 2,560, and in four 1,280. Despite this they exhibited some capacity to absorb the homologous coli agglutinins.

The homologous coli titre of MI-H6 serum was reduced fifty per cent after absorption with PB24. Absorption of VC-H3 serum by Y Ledingham reduced the homologous titre seventy-five per cent, even though this strain of *S. dysenteriae* agglutinated very weakly. Similarly, Karim Kahn and Y Ledingham removed seventy-five per cent of the homologous agglutinins from S-H6 serum. The T-H1 homologous titre was similarly reduced by PB24. Y Ledingham reduced the titre of G-H2 serum seventy-five per cent and PB24 fifty per cent. The A-H6 homologous titre was reduced seventy-five per cent after absorption by each of the three Flexner strains. The titre of the L-H2 serum was reduced fifty per cent by absorption with Y Ledingham. The titre of one serum, W-H12, was unaltered after absorption by the Flexner strains.

SUMMARY

The agglutination and absorption studies with patients' serum yielded evidence suggesting an immunological relationship between strains of *E. coli* and the type strains of *S. dysenteriae*.

Agglutination titres—S. dysenteriae vs. E. coli sera

E. COLI SERUM	TITRES UNABSORBED SERUM		SERUM ABSORBED WITH S. DYSENTERIAE VS. S. DYSENTERIAE		SERUM ABSORBED WITH S. DYSENTERIAE VS. HOMOLOGOUS E. COLI	
			Absorbing organism	Final titre corresponding organism	Absorbing Organism	Final titre for homologous E. coli
MI-H6.....	MI-H6	40,960				
	E. Led	40,960	E. Ledingham	20	E. Ledingham	20
	PB24	2,560	PB24	20	PB24	20,480
	KK	320	Karim Kahn	20	Karim Kahn	40,960
	Y Led	640	Y Ledingham	20	Y Ledingham	40,960
VC-H3.....	VC-H3	40,960				
	E. Led	40,960	E. Ledingham	20	E. Ledingham	20
	PB24	160	PB24	20	PB24	40,960
	KK	640	Karim Kahn	20	Karim Kahn	40,960
	Y Led	320	Y Ledingham	20	Y Ledingham	10,240
S-H6.......	S-H6	40,960				
	E. Led	40,960	E. Ledingham	20	E. Ledingham	20
	PB24	320	PB24	20	PB24	40,960
	KK	640	Karim Kahn	20	Karim Kahn	10,240
	Y Led	640	Y Ledingham	20	Y Ledingham	10,240
T-H1.......	T-H1	40,960				
	E. Led	40,960	E. Ledingham	20	E. Ledingham	20
	PB24	1,280	PB24	20	PB24	10,240
	KK	5,120	Karim Kahn	20	Karim Kahn	40,960
	Y Led	1,280	Y Ledingham	20	Y Ledingham	40,960
W-H12.....	W-H12	40,960				
	E. Led	40,960	E. Ledingham	20	E. Ledingham	20
	PB24	160	PB24	20	PB24	40,960
	KK	1,280	Karim Kahn	20	Karim Kahn	40,960
	Y Led	640	Y Ledingham	20	Y Ledingham	40,960
G-H2......	G-H2	40,960				
	E. Led	40,960	E. Ledingham	20	E. Ledingham	20
	PB24	640	PB24	20	PB24	20,480
	KK	640	Karim Kahn	20	Karim Kahn	40,960
	Y Led	320	Y Ledingham	20	Y Ledingham	10,240
A-H6.......	A-H6	40,960				
	E. Led	20,480	E. Ledingham	20	E. Ledingham	20
	PB24	1,280	PB24	20	PB24	10,240
	KK	2,560	Karim Kahn	20	Karim Kahn	10,240
	Y Led	2,560	Y Ledingham	20	Y Ledingham	10,240
L-H2.......	L-H2	40,960				
	E. Led	20,480	E. Ledingham	20	E. Ledingham	20
	PB24	320	PB24	20	PB24	40,960
	KK	640	Karim Kahn	20	Karim Kahn	40,960
	Y Led	640	Y Ledingham	20	Y Ledingham	20,480

Absorption of nine of these sera by the corresponding strain of
E. coli, in each instance, completely removed agglutinins for the
latter and for one or more of the S. dysenteriae strains as well.
Conversely, in the five sera studied, absorption by S. dysenteriae
removed both the dysentery and the coli agglutinins. Since it
was impossible to titrate the absorbing doses due to insufficient
serum, over-absorption may have been a factor in determining
these results. The frequency with which agglutinins for only
one of the antigens was affected renders this improbable.

Additional evidence of antigenic relationship was obtained from
the studies using monovalent dysentery anti-sera. All thirteen
strains of E. coli agglutinated to varying titre in these sera.
Absorption by the homologous antigen removed both the homolo-
gous dysentery and the heterologous coli agglutinins. Ab-
sorption with the individual strains of E. coli removed the
corresponding coli agglutinins and reduced the homologous titre
of the sera in varying degree.

Seven of the strains of E. coli, agglutinating in the unabsorbed
PB24 serum, reduced the homologous titre after absorption by
fifty per cent or more. This points to an antigenic component
common to these strains of E. coli and the PB24 S. dysenteriae.
The six strains which were agglutinated by the PB24 serum but
failed to absorb the homologous agglutinins lack this common
factor. Their agglutination, therefore, must be regarded as
non-specific.

Eight of the coli strains agglutinated in unabsorbed Karim
Kahn serum, and, when used to absorb the serum, reduced the
homologous titre by fifty per cent or more. Five strains failed
to absorb the homologous dysentery agglutinins and must, there-
fore, be considered antigenically unrelated to the Karim Kahn
Flexner bacillus. Agglutination of these organisms by this serum
must be classed as non-specific.

Nine of the E. coli strains, agglutinating in the unabsorbed
Y Ledingham serum, when used to absorb this serum reduced the
homologous titre by fifty per cent or more. Four failed to
absorb the dysentery agglutinins and are therefore unrelated to
the Y Ledingham Flexner bacillus. Agglutination of these or-
ganisms in this serum is consequently non-specific.

The results obtained with the E. Ledingham (Sonne) serum were striking. Six of the strains of *E. coli* agglutinated at full titre and completely absorbed the dysentery agglutinins. Two other strains, agglutinating at lower dilutions, likewise completely absorbed the homologous agglutinins. The remaining five strains, although agglutinating only in low dilutions, reduced the homologous serum titre by fifty per cent or more. These observations, therefore, point to agglutinogenic identity of six strains of *E. coli* and the E. Ledingham (Sonne) bacillus. The remaining seven strains appear to be closely related.

Confirmatory data were obtained from the studies of the thirteen monovalent anti-coli sera. The strains of *E. coli* fall into two groups, one unrelated to, and the second closely related to, if not antigenically identical with, the E. Ledingham (Sonne) bacillus.

The first group comprises five sera, none of which agglutinated E. Ledingham in high dilutions. Absorption of these sera with E. Ledingham in only one instance reduced the homologous coli titre by fifty per cent. The Flexner strains gave varying reactions. PB24 agglutinated to the full titre of the S-NH serum and completely removed the homologous coli agglutinins. The remaining four coli sera agglutinated the Flexner strains in lower dilutions. Absorption with one or more of these strains reduced the homologous titre of each serum by fifty per cent or more. These observations indicate an antigenic relationship, identical in one instance and close in a second, between the Flexner strains of *S. dysenteriae* and the five strains of *E. coli* comprising Group 1. It is interesting to note that non-specific agglutination of a Flexner bacillus may reach one-eighth of the titre of a monovalent coli anti-serum.

The behavior of the *S. dysenteriae* antigens in the eight monovalent coli sera comprising Group 2 points to agglutinogenic identity of the E. Ledingham (Sonne), and six of the strains of *E. coli*, and to a close relationship with two of the others. Despite the low titre agglutinations of the Flexner strains in these sera, the agglutinin absorption reactions noted indicate a serologic relationship between our Flexner antigens and seven of the strains of *E. coli* which may be of significance.

Comparison of the agglutination and agglutinin absorption activity of the four strains of *S. dysenteriae* and the thirteen strains of *E. coli* in the monovalent dysentery antisera and in the monovalent coli antisera reveals significant findings (table 10). These are not in exact conformity with the data obtained by the sera singly. Reciprocal agglutination and reciprocal absorption in the dysentery and the coli antisera demonstrate antigenic identity between E. Ledingham (Sonne) and five strains of *E. coli*. There is a close relationship with four additional coli strains.

TABLE 10

Evidence of relationship between E. coli and S. dysenteriae from reciprocal agglutination and reciprocal absorption experiments

E. COLI	S. DYSENTERIAE, PB 24		S. DYSENTERIAE, KARIM KAHN		S. DYSENTERIAE, Y LEDINGHAM		S. DYSENTERIAE, E. LEDINGHAM	
	Dysentery antiserum	Coli antiserum	Dysentery antiserum	Coli antiserum	Dysentery antiserum	Coli serum	Dysentery antiserum	Coli antiserum
MI-H6	0	+	0	0	+	0	+	+
VC-H3	0	0	0	0	0	+	+	+
S-H6	0	0	+	+	+	+	+	+
G-NH3	+	+	0	0	+	0	+	0
S-NH	+	+	+	+	+	+	+	0
W-NH7	0	+	+	+	+	0	+	+
W-H7	+	+	+	+	0	0	+	0
T-H1	+	+	+	0	+	+	+	+
A-H6	+	+	+	+	0	0	+	+
W-H12	0	0	0	0	+	+	+	+
L-H2	+	0	0	0	0	0	+	0
L-H1	0	0	+	+	0	0	+	+
G-H2	+	+	+	0	+	+	+	+

No such evidence of identity was found between the Flexner strains and the *E. coli*. Partial agglutination in the sera and significant reduction of homologous agglutinin titres after absorption was observed. Similar findings in both the coli and the dysentery antisera indicate a serologic relationship between six strains of *E. coli* and PB24 Flexner. Six coli strains likewise appear to be related to Karim Kahn Flexner, and six to Y Ledingham Flexner.

Five of the strains of *E. coli* are related to only one of the *S. dysenteriae* strains.   Three of the coli are related to two of the strains of *S. dysenteriae*, four are related to three of the *S. dysenteriae* strains, and one, A-H6, is related to all.

DISCUSSION

Four possible explanations may be advanced to account for these immunological cross-relationships between *Shigella dysenteriae* and *Escherichia coli*.   They may represent a major and minor agglutinin phenomenon dependent upon non-specific group agglutinins.   They may be regarded as an instance of paragglutination.   Unrecognized "R" dissociation of the cultures may have occurred with resultant development of common rough somatic antigens.   A true antigenic relationship may exist.

Complete removal of both homologous and heterologous agglutinins by absorption of the monovalent rabbit sera with their homologous antigens suggests a simple major and minor agglutinin phenomenon.   Heterologous absorption, however, presents strong evidence of a fundamental agglutinogenic similarity.   Moreover, the use of living antigens to absorb the sera insures maximal removal of both major and minor agglutinins. The use of formolized antigens for the agglutination reactions minimizes non-specific group agglutination.

The phenomenon of paragglutination is characterized by the agglutination of organisms other than the known etiologic agent in the serum of an infected individual.   The Weil-Felix reaction in typhus fever is a classic example.   As previously noted, various workers have observed that certain strains of *Escherichia coli* recovered from dysentery patients may be agglutinated in monovalent dysentery anti-sera.   Two explanations of this phenomenon have been advanced.   It is postulated that close association of *Escherichia coli* and *Shigella dysenteriae* within the patients intestinal tract alters the receptor apparatus of the *Escherichia coli* to resemble that of the *Shigella dysenteriae*. Arkwright (1931), however, considers that paragglutination is due to the presence of similar or identical "R" antigens.

The agglutinogenic cross-relationships which we have ob-

served might be classed as instances of paragglutination. This seems unlikely, if that phenomenon, in fact, is dependent upon alteration of receptor apparatus. The strains of *Escherichia coli* which we have examined were obtained from patients who never afforded cultural evidence of infection by *Shigella dysenteriae* despite repeated examination.

There remains the possibility that similar or identical "R" antigens may be present in the strains which we have been investigating. It is recognized that colony inspection and failure to flocculate in 0.85 per cent sodium chloride solution do not constitute positive proof of a pure "S" phase. The possibility of common "R" agglutinins has been avoided as carefully as present methods permit. None the less, partial "R" dissociation may have occurred and may account for the cross-relationships observed.

The general uniformity of results obtained by reciprocal agglutination and reciprocal absorption in the human sera, in the monovalent dysentery anti-sera, and in the monovalent coli antisera are strongly suggestive of a fundamental antigenic relationship between strains of *S. dysenteriae* and *E. coli*. Agglutination to full titre of a known monovalent anti-serum, and complete absorption of the homologous agglutinins of such a serum are commonly accepted as proof of antigenic identity of an unknown organism and the antigen used to produce the serum. Recognition of less close degrees of relationship is based upon partial agglutination and less complete absorption of homologous agglutinins. Judged by these criteria, subject to the potential error of unrecognized partial "R" dissociation, a true antigenic relationship exists between the four strains of *Shigella dysenteriae* and the thirteen strains of *Escherichia coli* studied.

The coli strains do not appear to be identical. Eight strains were non-motile. Ten were hemolytic on rabbit blood agar. Their respective immunological relationships will be reported subsequently.

We have previously called attention to the necessity for caution in the interpretation of the agglutination reaction for *S. dysenteriae* in chronic inflammatory disease of the colon. The

studies here presented give added emphasis.   They demonstrate that strains of *Escherichia coli* recovered from such patients may give rise to relatively high titre agglutinins for strains of *S. dysenteriae* Sonne and Flexner.   This leads inescapably to the conclusion that a positive agglutination reaction with these organisms does not constitute proof of homologous infection. Certain diagnosis of bacillary dysentery, therefore, rests upon, and only upon, recovery of *S. dysenteriae* on culture.

CONCLUSIONS

1. A close agglutinogenic relationship exists between certain strains of *Escherichia coli* and *Shigella dysenteriae* Sonne and Flexner.

2. The relationship between the strains of *Escherichia coli* and *Shigella dysenteriae* Sonne is closer than that between *Escherichia coli* and *Shigella dysenteriae* Flexner.

3. This relationship is such as to produce high titre heterologous agglutinins in the experimental animal.

4. These observations demonstrate that the agglutination reaction, unsupported by confirmatory bacteriologic evidence, does not constitute valid proof of infection by *Shigella dysenteriae* Sonne and Flexner.

5. The diagnosis of bacillary dysentery can be substantiated only by recovery of the organism.

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THE IMPLANTATION OF ORAL AND INTESTINAL STRAINS OF L. ACIDOPHILUS IN THE ALBINO RAT[1]

MICHAEL J. PELCZAR, JR.,[2] AND LUTHER A. BLACK

Department of Bacteriology, University of Maryland, College Park

During the past few years some investigators have ascribed to lactobacilli a definite rôle in the production of dental caries. A few have concluded from their studies that these lactobacilli were *Lactobacillus acidophilus* species, identical with those of intestinal origin, for example, Bunting (1937) states: "... dental caries is a specific bacterial disease and the specific organism involved is *L. acidophilus*." The principal exponents of these views include Howe and Hatch (1917), Rodriguez (1922), Bunting and Palmerlee (1925), Rosebury, Linton, and Buchbinder (1929), Howitt and van Meter (1930), Hadley (1933), Johnston, Williams, Anderson, Tisadall and Kaake (1936), Bunting (1937), and Jay (1937).

Other investigators are diametrically opposed to the foregoing claims as evidenced by many publications. Morishita (1929), Rettger (1932), Weinstein, Anderson and Rettger (1933), and Rettger, Levy, Weinstein and Weiss (1935), have concluded from their studies that the oral and intestinal strains of lactobacilli are distinctly separate groups of organisms, and they have not been able to demonstrate a striking correlation between the occurrence of caries and the presence of *L. acidophilus*.

In view of these contradictory opinions and since reported

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[1] Based on a portion of the thesis submitted by the senior author to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Master of Science.

[2] Present address, Department of Bacteriology, State University of Iowa, Iowa City.

results indicate that cultural, morphological, biochemical and serological characteristics afford a poor basis for differentiation of these lactobacilli, the present investigation was undertaken to determine whether the organisms of either oral or intestinal origin differed in their ability to become implanted in the intestinal tract of white rats.

EXPERIMENTAL

Methods

Cultures—type and source. A. *Oral or dental strains:* Nos. 14, 18, and 19 were obtained from L. F. Rettger, labelled S₅, Flynn, and Rosebury respectively. Nos. 60, 61, 94, and 97 were obtained from H. R. Curran.[3] All of these cultures were of the smooth (Y) type. B. *Intestinal strains:* No. 64 was obtained from Curran, originally isolated from acidophilus milk, nos. 15 and 20 from Rettger,[4] and nos. 1 and 9 from R. P. Meyers. All of these were rough (X), filamentous colony types. Nos. 10 and 12 were received from J. M. Sherman, K from C. W. England, and K4y from N. Kopeloff. These were smooth (Y) intestinal types. C. *Lactobacillus bulgaricus:* Only one culture was used. This was obtained from Sherman, originally from Rettger.

Preparation of milk culture. The milk culture used for feeding was prepared by the inoculation of skimmed milk, sterilized by autoclaving. Cultures used for inoculum in the preparation of the fermented milk and for daily feeding were forty-eight hours old. Plate counts were made of each fermented milk several times during the course of this work and with but two exceptions these indicated that the viable organisms in each preparation were several hundred million per cc.

Animals and diet. The implantation of the organisms was determined by feeding experiments conducted with male and female albino rats. Before use in the experiments the rats were maintained on a ration of commercial dog pellets which kept them

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[3] Originally from Enright, his nos. 144 and 42, the Morishita collection no. 13, and Kulp, his S b, respectively.
[4] The Wickerham and Cohen starter strains respectively.

in an apparently healthy condition.   Two weeks previous to the administration of a culture, each rat was placed in a separate cage and fed solely upon 10 grams of ground beef daily in addition to water.   At the end of the two-week period, the intestinal flora, as determined by microscopic examination of gram-stained smears and plate cultures from fecal specimens, was composed almost entirely of gram-negative bacteria.   After this period, the rats were fed approximately 5 cc. of the lactobacilli preparations daily (one strain for each rat), in addition to the ground beef.   As controls one rat was fed meat only, and another rat 5 cc. of sterile skimmed milk daily in addition to meat.   The feeding continued for three weeks, after which five of seventeen rats were sacrificed for bacteriological examination of intestinal contents (table 2). The remaining rats were then fed a diet of meat (10 grams), sterile skimmed milk (5 cc.), and lactose (1 gram) for two weeks during which time fecal examinations were made.

Collection of feces and intestinal contents.   Fecal specimens were collected regularly at three-day intervals while feeding cultures, and after 3, 6 and 14 days during lactose feeding.   Specimens were collected as nearly as possible under aseptic conditions into sterile petri dishes.

The entire intestinal tract was removed from each rat sacrificed, sections selected from the duodenum, ileum, caecum, and colon, and the contents ejected into a sterile 9 cc. physiological saline dilution blank.

Bacteriological examination.   A portion of each fecal specimen, approximately one-fourth of a gram, was disintegrated into a uniform suspension in 9 cc. of sterile physiological saline.   The collection of intestinal specimens in 9 cc. blanks sufficed for the initial dilution.   Smears were made from these original suspensions and gram-stained, and further dilutions of 1:1,000, 1:10,000, and 1:1,000,000 were prepared for plating.   While the dilutions were not exactly quantitative (since the original sample was approximate) this did not interfere with the results inasmuch as determination of the number of bacteria per gram of feces was not attempted.   Identification of *L. acidophilus*, and determination of its approximate percentage of the total bacterial flora were the primary objectives.

The plating medium used was that suggested by Rettger *et al.* (1935) composed of tomato juice, peptonized milk, neopeptone, and yeast extract.   All plates were incubated in an atmosphere of 10 per cent carbon dioxide at 37°C. for 3–5 days.   Following incubation, the plates containing the most suitable number of colonies for examination were selected for further study.   By means of a dissecting microscope the various colony types were examined and their description recorded, together with the approximate percentage of each type.   Gram stains were made of each colony type, and tubes of litmus milk were inoculated from colonies resembling those of lactobacilli.   Litmus milk tubes showing reactions characteristic of the lactobacilli were held for identification studies.   Sometimes it required several transfers before a typical reaction occurred.   Tubes in which curdling and reduction of litmus milk did not occur were discarded.

Identification of cultures.   It was not our aim to make a detailed study of the cultural and biochemical characteristics of all strains employed.   Our primary objective was to determine a few characteristics of each strain before implantation and then repeat these same tests upon *acidophilus-like* organisms which were isolated from the feces.   In this manner we could determine whether the recovered organism was the same one which was being administered orally.   For this purpose the morphology, colonial characteristics, ability to ferment maltose, mannitol, sucrose and raffinose, and the phenol resistance of each original strain, as well as of all isolated strains, were recorded.   Lipolytic activity, although not determined in this study, has been suggested for differentiation of closely related strains of lactobacilli, and Sabine (1937) indicated that this was valuable in clinical work to demonstrate that recovered strains were identical to administered strains.   •

Fermentation studies were carried out in precisely the same manner described by Curran, Rogers, and Whittier (1933) except that a proteose-peptone yeast-extract broth was used as a basic medium instead of casein-digest broth.   In preliminary experiments this proteose-peptone yeast-extract broth was shown capable of supporting good growth of both oral and intestinal strains of

lactobacilli. Its use was advantageous, both in simplicity of preparation and in uniformity of composition of different batches of medium, which may be variable with casein-digest broth. Phenol tolerance was determined as described by Kulp (1929) and employed by Curran, Rogers and Whittier, except that agar shake cultures were substituted for plate cultures.

Results

An example of the detailed manner in which all fecal specimens were examined is given in table 1, such results being recorded for each rat used. Although the examination of the stained smears of fecal specimens along with plating and macroscopic and microscopic examination of resulting colonies were useful in following the change in fecal flora, we were interested primarily only in identification of lactobacilli strains. Table 1 also illustrates the detailed plan used in partially identifying lactobacilli recovered after implantation, some additional characteristics used for this purpose being listed in table 3. This is mentioned, particularly, since it is our opinion that the process of identifying lactobacilli in some earlier studies of implantation may have been done hastily and perhaps inadequately.

A summary of the percentages of lactobacilli present in each specimen examined by the above-mentioned procedure is given in table 2. It will be noted that two figures are given for each analysis, the first indicating the *entire Lactobacillus* content of the feces irrespective of type, while the second percentage refers to the *type administered* to that particular animal, as determined by certain characteristics apparently of value in differentiation as summarized in table 3.

Controls. The rat (no. 2) subsisting on the basic diet (10 grams of ground beef) had a fecal flora in which gram-negative organisms predominated. Lactobacilli could not be isolated by cultural methods. Rat 1 which received 5 cc. of sterile skimmed milk in addition to ground beef yielded lactobacilli in the feces after two weeks, and later, when the animal received lactose, these organisms were predominant, almost to the exclusion of all other types.

Oral strains. Rats fed oral strains of lactobacilli and the one

TABLE 1

Typical results of microscopic and cultural examination of fecal specimens from a white rat receiving L. acidophilus culture
Rat 13.   Culture 1 (intestinal (X) strain).   Diet 10 grams beef + 5 cc. of milk culture

DAYS ON DIET	FECAL SUSPENSION		COLONIES		ORGANISMS		
	Stain, shape	Description	Description	Per cent	Stain, Shape	Description	L.A. per cent
3	Gram + rod	Long-single, pairs	Subsurface filamentous	80	Gram + rod	Long, thin-single, short chains	80
	+ rod	Short-single, chains	Subsurface Elliptical smooth	15	+ rod	Short, single-pairs, chains	
	− rod	Short, single	Surface circular, large	5	− rod	Short, single	
	+ cocci	Single and pairs					
6	+ rod	Long-single, pairs, chains	Subsurface, very filamentous	95	+ rod	Long, thin-single, short and long chains	95
	+ rod	Short-single, pairs, chains	Subsurface, circular and elliptical	5	+ rod	Short-single, chains	
	+ cocci	Single, pairs (large)					
	− rod	Single, long and short					
9	+ rod	Long-single, chains	Subsurface, extremely filamentous	99	+ rod	Long, thin-single, chains	99
	+ rod	Short-single, chains	Subsurface, circular and elliptical	1	+ rod	Short-single, chains	
	++ cocci	Single-pairs, chains					
12	+ rod	Long, thin-single chains	Subsurface, extremely filamentous	99	+ rod	Long, thin-single, chains	99
	++ cocci	Short-single, chains	Subsurface, circular and elliptical	1	+ rod	Short, single-pairs, chains	
		Single and pairs					
	− rod	Single, short					
15	+ rod	Long, thin-single, chains	Subsurface, extremely filamentous	90	+ rod	Long, thin, single and chains	90
	++ cocci	Short-single, chains	Subsurface, circular and elliptical	10	+ rod	Short-single, chains	
		Single, pairs					
	− rod	Short, single					

No.	L.A.	Morphology	%	Colony	L.A.	Morphology	%
18	+ rod	Long, thin-single, pairs, chains	99	Subsurface, extremely filamentous	+ rod	Long, thin-single, chains	99
	++ rod	Short-single, chains	1	Subsurface, circular, elliptical	+ rod	Short-single, chains	
	+ cocci	Single, pairs (large)					
	− rod	Short-single					
21*	+ rod	Long, thin-single, pairs, chains	99	Subsurface, extremely filamentous	+ rod	Long, thin-single, chains	99
	+ rod	Short-single, chains	1	Subsurface, circular and elliptical	+ rod	Short-single, chains	
	+ cocci	Single, pairs					
24	+ rod	Long, thin-single, short chains	80	Subsurface filamentous	+ rod	Long, thin-single, chains	100
	+ rod	Short-single, short chains	20	Surface and subsurface circular, elliptical	+ rod	Short-single, pairs	
	+ cocci	Large-single, pairs					
27	+ rod	Short-single and short chains	50	Surface and subsurface circular, elliptical	+ rod	Short-single, short chains	45
	+ rod	Long, thin-single, short chains	45		+ rod	Long, thin-single, chains	
	+ cocci	Large-single, pairs, short-single (very few)	5	Subsurface filamentous; surface, circular, large	− rod	Short-single	
35	+ rod	Short-single, chain	80	Surface and subsurface circular and elliptical	+ rod	Short-single, pairs and short chains	10
	+ rod	Long, thin-single, pairs	10		− rod	Short-single	
	+ cocci	Large-single, pairs	10	Surface, circular, moist; subsurface filamentous	+ rod	Long, thin-single and pairs	
	− rod	Short-single					

L. A. Percentage of *L. acidophilus* of the strain present in the milk culture administered.
* Last day of administration of milk culture—*L. acidophilus* feeding replaced by 5 cc. of sterile skimmed milk and one gram of lactose.

TABLE 2

Percentage of Lactobacilli in the feces of albino rats and the percentage of the total comprised of organisms administered orally in milk cultures

(Per cent Lactobacilli as determined from tomato agar plates)

DIET: GROUND BEEF PLUS	RAT NO.	DAYS ON DIET									
		3	6	9	12	15	18	21*	24	27	35
		A/A'	A/A'	A/A'	A/A'	A/A'	A/A'	A/A'	A/A'	A/A'	A/A'
Controls: Sterile milk	2	0/0	0/0	0/0	0/0	0/0	0/0	0/0†			
	1	0/0	0/0	0/0	0/0	40/0	40/0	40/0	80/0	80/0	90/0
Dental strains:	8	100/0	85/0	90/0	95/0	95/0	85/0	90/0†			
	18	0/0	80/0	90/0	90/0	90/0	90/0	80/0	90/0	90/0	90/0
	19	0/0	40/0	90/0	95/0	95/0	95/0	95/0†			
	60	0/0	20/0	90/0	80/0	90/0	90/0	90/0	90/0	80/0	90/0
	61	0/0	20/0	90/0	90/0	90/0	90/0	85/0	70/0	80/0	90/0 †
	94	0/0	90/0	90/0	100/0	100/0	100/0	100/0†			
	97	80/0	90/0	80/0	80/0	90/0	90/0	80/0	80/0	80/0	90/0
L. bulgaricus: B	5	10/0	80/0	85/0	80/0	85/0	90/0	90/0†	80/0	80/0	90/0
Intestinal strains (rough): 1	13	95/80	100/95	100/99	100/99	100/90	100/99	100/99	100/80	95/45	90/10
9	19	60/10	95/25	100/60	100/80	100/80	100/80	100/80	100/25	95/40	70/30
15	18	75/25	100/95	100/98	100/100	100/100	100/100	100/100	100/50	100/45	65/5
20	15	90/30	100/95	100/90	100/90	100/100	100/100	100/100	100/80	100/25	90/15
61	12	95/0	95/0	100/0	100/0	100/0	100/0	100/0	100/0	90/0	90/0
Intestinal strains (smooth): 10	16	70/20	70/20	90/30	100/40	100/70	100/90	100/90	100/60	80/30	70/0
12	14	30/0	90/40	95/35	100/40	100/55	100/60	100/50	80/20	50/0	60/0
K	17	90/30	95/45	95/35	95/35	95/45	95/35	95/35	100/20	75/5	80/0
K4y	11	60/30	60/30	90/70	90/80	100/90	95/85	100/80	80/30		90/0

* Last day of administration of milk culture—diet changed to sterile skimmed milk (5 cc.) and ground beef (10 grams), plus lactose (1 gram).

A—Percentage of total fecal flora composed of lactobacilli.

A'—Percentage of total fecal flora present representing the *strain administered* in the milk culture.

† Sacrificed for bacteriological examination of intestinal contents.

rat receiving a culture of *L. bulgaricus* exhibited the same characteristic trend with respect to intestinal transformation. In each case, without exception, lactobacilli predominated after the first few days of feeding. However, the lactobacilli which were isolated from these rats differed slightly in their colonial characteristics from the strains which were being fed. In additions, the same organism appeared in the feces of the rat receiving *L. bulgaricus* and a control rat receiving sterile skimmed milk. This made it evident that the recovered organisms were not the oral strains being fed to the rats, but instead, some other lactobacilli, apparently a rat strain initially present in the intestinal tract and stimulated by milk in the diet. As will be shown later, by comparing the cultural and physiological characteristics of the organisms fed and isolated, we were able to conclude that the oral strains were not present in the feces. Similarly *L. bulgaricus*, was never recovered from the feces of the rat receiving this culture. At the end of three weeks, when lactobacilli culture feeding ceased, and the diet of five rats of this group consisted of ground beef, sterile skimmed milk, and one gram of lactose, there was practically no change in the intestinal flora of those rats which had previously received lactobacilli cultures. The control rat (no. 1) which had been fed meat and sterile skimmed milk, and now received lactose in addition, yielded a marked increase of lactobacilli.

The remaining five rats of this group, the one fed ground beef, the one fed *L. bulgaricus*, and three which had received oral strains of lactobacilli were sacrificed. Upon examination of specimens from the duodenum, ileum, caecum and colon of each animal, organisms of the original strains could not be isolated although lactobacilli, apparently rat strains and identical with those found in previous examinations of the feces were recovered. With the exception of duodenal specimens where organisms were relatively scarce and in one case absent, bacteriological examinations of sections of the intestinal tract of sacrificed animals yielded the same results as those reported from examinations of the feces of live animals.

Intestinal strains. With one exception (culture 64) the rough

intestinal organisms were recovered from the corresponding rats to which they were fed. Apart from the one exception, the rough strains appeared in feces in great numbers after the first few days of feeding almost to the exclusion of all other bacteria. The smooth strains did not show such predominance, although in most cases after two weeks of feeding they constituted the majority of the bacteria present. Lactobacilli of the type isolated from rats receiving oral strains were also isolated. As can be seen from table 2, the intestinal flora of each rat in the group receiving intestinal strains of *L. acidophilus* was comprised almost entirely of lactobacilli, including both the administered intestinal strain and the rat strain apparently initially present in the intestinal tract. When *L. acidophilus* culture feeding was replaced by lactose and sterile skimmed milk there was a gradual disappearance of the strain which had been administered, with a gradual increase of the rat intestinal type. After two weeks without culture feeding the smooth intestinal strains which had been fed were no longer present in the feces, while the rough strains, with the exception mentioned previously (culture 64), were present to a very small extent. In each instance after cessation of culture feeding the rat intestinal type of lactobacilli eventually predominated over the strains fed.

Cultural characteristics. The morphology of all organisms varied considerably, which is characteristic of the *Lactobacillus* genus. Some of the rods were extremely short having the appearance of ovoid cells while others were slender and long, appearing single, in pairs and in chains. The rat strains of lactobacilli isolated were nearly always short to medium sized rods. Colonies of smooth intestinal and smooth oral types could not be differentiated. The rat strains also produced colonies which were smooth, but more dense, white, smaller, and always had an entire edge, while colonies of the rough intestinal strains were extremely filamentous. All strains reduced and curdled litmus milk after 24 or 48 hours. All strains of the oral and intestinal groups fermented maltose, sucrose (with one exception) and mannitol (with two exceptions). These exceptions occurred among the oral strains. The reaction on raffinose was variable. Those

TABLE 3

Certain cultural and biochemical characteristics of strains of Lactobacilli fed and strains isolated from the feces of albino rats

CULTURE NUMBER	RAT NUMBER	ORIGINAL CULTURES FED						CULTURES ISOLATED					
		Colony type	Sucrose	Maltose	Mannitol	Raffinose	Phenol tolerance	Colony type	Sucrose	Maltose	Mannitol	Raffinose	Phenol tolerance
Controls	2	(Basic diet)						(No lactobacilli isolated)					
	1	(Basic diet plus skimmed milk)						S	+	−	+	−	1:400
L. bulgaricus B	5	R	+	−	−	−	1:300	S	+	+	+	−	1:200
Oral strains (L. acidophilus):													
14	8	S	+	+	+	+	1:500	S	+	−	+	−	1:300
18	9	S	+	+	+	+	1:600	S	+	−	+	−	1:400
19	10	S	+	+	+	+	1:400	S	+	−	+	−	1:400
60	6	S	+	+	+	−	1:500	S	+	−	+	−	1:200
61	3	S	+	+	+	+	1:500	S	+	+	+	−	1:200
94	7	S	−	+	−	−	1:600	S	+	−	+	−	1:500
97	4	S	+	+	−	−	1:400	S	+	−	+	+	1:300
Intestinal strains (L. acidophilus):													
1	13	R	+	+	+	+	1:200	R*	+	+	+	+	1:200
								S	+	−	+	+	1:500
9	19	R	+	+	+	−	1:400	R*	+	+	+	−	1:300
								S	+	−	+	−	1:600
15	18	R	+	+	+	+	1:300	R*	+	+	+	+	1:300
								S	−	−	+	+	1:400
20	15	R	+	+	+	+	1:200	R*	+	+	+	+	1:200
								S	+	−	+	−	1:400
64	12	R	+	+	+	−	1:400	S	+	−	+	−	1:400
10	16	S	+	+	+	−	1:200	S*	+	+	+	+	1:200
								S	+	−	+	+	1:300
12	14	S	+	+	+	+	1:300	S*	+	+	+	+	1:200
								S	+	−	+	−	1:500
K	17	S	+	+	+	+	1:200	S*	+	+	+	+	1:200
								S	+	−	+	−	1:200
K4y	11	S	+	+	+	+	1:200	S*	+	+	+	+	1:200
								S	+	−	+	−	1:400

* Has the same characteristics as the culture originally fed, indicating implantation of the original strain.

strains isolated which were of rat origin fermented sucrose and mannitol with only one exception while the reaction on maltose and raffinose was variable. Intestinal strains were much more tolerant to phenol than the oral strains, being able to initiate growth in concentrations of 1:400 or greater. Strains of rat origin, although not as resistant to phenol as the stock intestinal strains, were slightly more tolerant than the oral strains used.

These cultural characteristics are presented in detail in table 3, wherein the characteristics of the strain administered orally is compared to those of *acidophilus-like* organisms found in the feces of the same rat. In this way it could be ascertained whether the organism isolated from the feces of a particular rat was the same one which had been fed to that rat.

As already shown, fermentation reactions alone were inadequate for classification of the strains but they were of real value, along with cultural and other characters, in determining the characteristics of a known strain before implantation. By repetition of these tests upon organisms isolated after culture feeding we could determine with considerable certainty whether or not the recovered organisms were identical with those administered. Colonies of the rat strains (always smooth) did differ in size, color and contour from the smooth human types and we believe the constancy of these characteristics and the appearance of the organisms in control rats, along with the fermentation reactions described above, warrant designation of these lactobacilli as rat strains.

DISCUSSION

The implantation of *L. acidophilus* in the intestinal tract of man and rats has been repeatedly demonstrated. Since results of the present investigation show that none of the oral strains used were implantable it seems that some differences must exist between the lactobacilli of oral and intestinal origin. This is in accord with the results of Rettger and associates, Curran, Rogers and Whittier, and Ulicny, who have reported differences between these two groups with respect to cultural characteristics, type of lactic acid produced, and the quantitative utilization

of lactose, respectively, but different from the conclusions of those who maintain that both groups are comprised of the same organisms. Considering the many similarities which exist between the oral and intestinal lactobacilli and the fact that many of the differences are quantitative in nature, we would be inclined to class the oral strains as atypical species and not the true central type, *L. acidophilus*. While no studies were made of these organisms from the standpoint of the etiology of dental caries, we are inclined to be of the same opinion as Rettger, Levy, Weinstein, and Weiss who maintain that there is still a lack of sufficient evidence to show that the intestinal types are of significance in dental caries.

The failure to implant one of the intestinal strains (64) is significant but not necessarily surprising. Except for the inability of this organism to become established in the intestinal tract of rats, its characteristics conformed to those of other strains of *L. acidophilus* which were implanted. This is significant in view of the similarity between the two species, *L. acidophilus* and *L. bulgaricus*, the latter considered not implantable. Although some have separated these species on the basis of maltose fermentation, the recent work of Curran and associates hardly substantiates such a separation. Recently Kopeloff and Kopeloff (1937) reported that R forms of both *L. acidophilus* and *L. bulgaricus* produce inactive lactic acid, while the smooth types of both produce dextro-rotary acid. Admitting the intimate relationship between these organisms, it is questionable whether our culture no. 64 should be considered an atypical strain of *L. acidophilus* or whether it belongs to the *L. bulgaricus* species. Perhaps organisms described as *L. bulgaricus* may be in reality unimplantable *L. acidophilus*. We are of the opinion that culture no. 64 is an atypical strain of *L. acidophilus* inasmuch as with other bacteria it is not unusual to find one species of a group deviate so from the central or typical type.

The appearance in the feces of lactobacilli of a type other than those fed was to be expected since they may be normal inhabitants of the intestinal tract, their predominance depending upon the composition of the diet. Porter, Weinstein, and Rettger (1938)

in an investigation to ascertain the bacterial flora of the stomach, segments of the small intestine and cecum of white rats, found lactobacilli present in large numbers throughout. Eppright, Valley and Smith (1937) have shown that salts of calcium and phosphorus, in addition to carbohydrates, were essential for the maintenance of an aciduric flora. Since milk contains all of these we would expect it to favor the development of those aciduric organisms already present as well as those which were being fed, providing the organisms were implantable, and this appeared to occur in our experiments. However, acidophilus milk was much more effective than sterile skimmed milk in increasing the number of those aciduric organisms originally present in the intestinal tract.

Whether the aciduric organisms isolated from rats should be classified as *Lactobacillus acidophilus* or *Lactobacillus bifidus* is another debatable question. Rettger and others have reported isolation and identification of both *L. acidophilus* and *L. bifidus* from rat feces. Other than differences in carbohydrate reactions which are of questionable value for separating members of this genus, the organisms isolated in our study were very much like the smooth (Y) type of *L. acidophilus* and hence we have designated them as such. Possibly some could have been placed in the so called *L. bifidus* groups, except that results already published may not warrant the establishment of two distinct species for organisms so closely related. Weiss and Rettger (1934) (1938) have emphasized the similarity of these organisms and suggest that *L. bifidus* from breast-fed infants and rats be considered a variant of the species *L. acidophilus*.

Another point of interest is the possible significance of aciduric organisms normally present in the intestinal tract. Since these species may be made to predominate by regulation of the diet it would seem that when they are present it might be superfluous to administer other foreign aciduric bacteria. However, in these studies the administration of *L. acidophilus* milk possessed a distinct advantage over sterile milk in stimulating the normal aciduric flora.

Finally, this investigation emphasizes the need for a method of

examining commercial acidophilus preparations beyond mere identification of organisms. Our results indicate that there may exist rough (X) types of *L. acidophilus* which are not implantable in white rats, although they are typical in all other respects. This is in agreement with other investigations which have indicated that some strains of *L. acidophilus* vary in their ability to become acclimated to the intestinal tract. Hence, it seems that the important features of an analytical procedure for acidophilus preparations should be to establish the identity of the organisms and to determine their implantation.

SUMMARY AND CONCLUSIONS

Of the seven oral or dental strains of lactobacilli employed, none were implantable in the intestinal tract of white rats by the procedure used. On the other hand, four of five rough intestinal strains and all of the five smooth intestinal strains were implantable.

Rough intestinal *Lactobacillus acidophilus* organisms (with the exception of one strain) were more readily implanted and persisted for a longer time following the cessation of feeding the organisms than did smooth strains. The one culture of *Lactobacillus bulgaricus* used was not implantable which is in accord with previous studies.

With the exception of the duodenal contents, which contained relatively few organisms, other specimens from sections of the intestinal tract of the animals sacrificed gave results upon microscopic examination and cultivation similar to those obtained with fecal samples.

Fermentation of sucrose, maltose and raffinose by intestinal strains, sucrose and maltose by dental strains and sucrose and mannitol by rat strains occurred with few exceptions. The remaining fermentation reactions were irregular and, thus, fermentation reactions alone could not be used for separation of strains into groups, although in individual instances fermentation results aided in identification of a given strain. The intestinal strains exhibited a slightly higher tolerance to phenol than did

the dental strains, and this was the only biochemical characteristic used which correlated with the results on implantation.

Lactobacilli, apparently rat strains of *Lactobacillus acidophilus*, were isolated from each rat receiving lactobacilli milk cultures as well as from a control rat receiving sterile skimmed milk. This emphasizes the necessity of controlling implantation experiments adequately to insure that the strain isolated is actually the strain administered, especially when smooth strains of lactobacilli are employed.

The results emphasize the errors that are likely to occur in identifying aciduric organisms from the oral cavity as *Lactobacillus acidophilus*. Biochemical differences between intestinal and oral strains have been shown to exist by other investigations, while this work points out the inability of the oral strains studied to become implanted in the intestinal tract of rats. Such evidence may not justify designation of oral strains of aciduric organisms as *Lactobacillus acidophilus* since implantation is a generally accepted characteristic of this species.

The results also indicate the need for improved methods of examining *Lactobacillus acidophilus* preparations to include not only identification of the organisms but in addition whether or not they can be implanted.

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A STUDY OF THE HEMORRHAGIC SEPTICEMIA PASTEURELLAE

CARLOS T. ROSENBUSCH AND I. A. MERCHANT

Department of Veterinary Hygiene, Division of Veterinary Medicine, Iowa State College, Ames, Iowa

Early investigators depended for the diagnosis of hemorrhagic septicemia entirely upon the lesions and the bipolar staining and pathogenicity of the organisms encountered as etiologic agents. It is evident, therefore, that in early references, organisms of widely divergent characteristics have been included in the *Pasteurellae* group.

The first report of an organism of this group was by Rivolta in 1877 following his study of fowl cholera. This investigation was followed by several others, establishing a host of organisms as the causative agents of the various hemorrhagic septicemia diseases. Hueppe, in 1886 was the first to observe the close relationship of the various organisms of the group when he used the name *Bacterium septicaemia-hemorrhagica* for the etiologic agents of fowl cholera, cattle, rabbit, and swine septicemia. Kitt, however, in 1885 used the term *Bacterium bipolare-multocidum* to embrace the organisms causing the disease in cattle, swine, deer, horses, sheep, and goats. These two authors were the first exponents of the unicist school. The work of Lignières, however, is probably the basis of the zoologic species classification of the Pasteurellae, even though he recognized the close cultural and biochemic similarities of the various group constituents. Lignières' classification was based almost entirely on the isolation history of the various organisms. This classification has prevailed up to the present, although experimental evidence has pointed to its inadequacy as a method of subdividing the genus.

Baumgarten (1911) was one of the first investigators to doubt the value of the zoologic classification, basing his objection on the

experimental cross-pathogenicity of the various groups or species. Similar conclusions regarding experimental and field cross-pathogenicity, as well as cross-immunity, have been reported by the following investigators: Mohler and Eichhorn (1913), Hutyra (1925), Migge (1933), and Manninger (1934).

Further evidence against the zoologic classification was provided by studies of the cultural, biochemic, and serologic characteristics of the various components of the group. A general tendency to doubt the value of Lignières' classification has resulted, since there is a decided similarity between the various strains and none of the strain differences correspond to the zoologic species subdivision.

On the basis of cultural, biochemic and serologic characteristics, the Pasteurellae have been divided into a typical group and an atypical group by several investigators. Organisms belonging to the latter group isolated from cattle and sheep have been studied by Jones (1921), Spray (1923), Jorgensen (1925), Eddington (1930), Newsom and Cross (1932), and others. Newsom and Cross, in 1932, studied this group in detail and reported it as identical to Jones' Group I, because the two groups are similar in pathogenicity, hemolysis, absence of indol formation, and fermentation of lactose, maltose, inositol, dextrin, and raffinose. These last investigators placed the atypical strains in a separate species which they called *Pasteurella hemolytica*.

The typical group has been studied by investigators in various countries and subdivided into several subgroups or types, which, unfortunately, have not been correlated sufficiently for their classification status to be established. Some of the investigators responsible for the subdivision of this group, which contains most of the hemorrhagic septicemia Pasteurellae are: Koske (1927), Roderick (1922), and Zaisen (1934), all of whom resorted to complement-fixation differences, and Cornelius (1929), Ochi (1934), Yusef (1935), and Khalifa (1936) on the basis of agglutination, agglutinin-absorption, and precipitin-absorption reactions. Khalifa, in 1934, was the first investigator to correlate the serologic results with the *fermentation of xylose, arabinose and mannitol*.

Culture variations have been observed in the Pasteurellae by many investigators; however, few of the results have been correlated. The earliest report was by Manninger (1919) who described an avirulent, uncapsulated, highly immunogenic culture-variant of avian origin.

Other supposedly rough variants were described by De Kruif (1922) as virulent "D", and avirulent, rough "G" types. Webster and Burn (1926), added to these an "I" or intermediate type and a more stable "M" or mucoid type. Anderson, Coombes, and Mallick, in 1929, initiated the use of "S" and "R" to replace the "D" and "G" types of De Kruif. Brigham and Rettger (1935) used the same terminology and, in addition, recognized the occurrence of "I" or intermediate forms.

Other reports describing cultural variations dealt with fluorescence. Webster and Hughes (1929) described three types on the basis of fluorescence, pathogenicity, and agglutinability. The fluorescent form was highly pathogenic and of poor agglutinability, whereas the non-fluorescent form was of lower pathogenicity. The third type was an intermediate. Morch and Krogh-Lund (1931) and Ochi (1933) reported types that seem to be similar to the three types described above. Cornelius, in 1931, also found a similar change, but he used the terms, "I" form, for the less stable, fluorescent and poorly agglutinable type, and "A" form for the stable non-fluorescent and highly agglutinable type. This author stated that he could obtain "A" forms from his "I" forms, by subjecting the cultures to unfavorable environmental conditions, but he could not do the opposite, for they reverted very rapidly to the original "A" form.

EXPERIMENTAL MATERIALS AND PROCEDURE

One hundred and fourteen strains were studied, but due to the lack of space[1] results for only 44 representative strains are presented in tables 1 and 3. The isolation history of many of the strains, other than the species from which they originated,

[1] For additional complete data refer to C. Rosenbusch's Master's Thesis on the "Biologic and Serologic Relationships of the Hemorrhagic Septicemia Pasteurellae" deposited at the Library of the Iowa State College, Ames. Iowa.

was not available. The origin of the 114 strains studied was as follows: 38 avian, 22 bovine, 18 ovine, 15 porcine, 7 buffalo, 7 equine, 4 rabbit, and one each of deer, cat, and mink. Of the 44 cultures discussed in this paper[1] the source and original numbers are as follows:

1. Strains: 153 (1336-Jones), 159 (Woodbury), 161 (Mountain sheep), 164 (52B), 165 (4277-Jones), 168 (54), 169 (33), 175 (18A), 178 (Hereford), from Dr. I. E. Newsom, Ft. Collins, Colorado.
2. Strains: 104 (8-65), 106 (8-75), 109 (8-58), 116 (8-71), 120 (8-66), 122R (8-54), 130 (8-76), 134 (8-64), 138 (8-70) from the Jensen-Salsbery Laboratories, Kansas City, Missouri.
3. Strains: 70 (170), 84, 95, 217M (117), 257, 335, 642 from the Pittman-Moore Laboratories, Indianapolis, Indiana.
4. Strains: 150, 226, 412, 1525, 4300 from the Fort Dodge Serum Co. Laboratories, Fort Dodge, Iowa.
5. Strains: 232, 234R, 236, 242R, 243 from the Norden Laboratories, Lincoln, Nebraska.
6. Strains: 33R (N33), 590M (2590), 779 (H779) from the United States Bureau of Animal Industry Laboratories, Washington, D. C.
7. Strains: 31, 57, 1932 from the Lederle Laboratories, Pearl River, New York.
8. Strain: 886 from Dr. H. D. Marsh, Bozeman, Montana.
9. Strains: 35M, R, T, 70R from miscellaneous sources.

Morphologic and cultural technic

Preparations stained by dilute carbol-fuchsin and Giemsa stains were examined to detect capsules. A basic medium containing 0.5 per cent proteose-peptone (Difco) and 0.5 per cent sodium chloride, 0.02 per cent di-potassium-phosphate, and 0.01 per cent magnesium sulphate at a pH of 7.2–7.4, or a buffered pepsin-digested beef-infusion medium was used for cultural purposes. A medium containing beef-extract broth, 1.2 per cent agar and 20 per cent serum was used for stock cultures, colony studies, and dye bacteriostasis.

Growth characteristics in broth were observed at daily intervals for the detection of variants. Motility was studied after 24

hours of growth in the same broth cultures. Hemolysis was determined on dilute horse-blood agar plates and recorded after 18 to 36 hours. Bile solubility tests were made by the addition of 0.5 to 1 cc. of heat-sterilized sheep bile to 24-hour cultures.

Dye bacteriostasis tests were carried out in a series of dilutions between 1:10,000 and 1:100,000 of crystal violet, basic fuchsin, thionine, pyronine B, malachite green, and brilliant green. Several cultures were inoculated on the same plate and care was taken to prevent the use of too great bacterial inocula which would otherwise allow growth by superposition of organisms. Readings were taken at daily intervals for five days.

Biochemic and pathogenesis technic

A total of 15 carbohydrates, 6 alcohols, and 1 glucoside were used to study the saccharolytic properties of the organisms. The fermentation tests were made in a medium composed of one-per-cent of the sugar in basic broth with one-per-cent of Andrade's indicator. The media were filter-sterilized through a W Berkefeld candle. Readings were taken daily for 10 days and then at 10-day intervals until the thirtieth day. The fermentation tests were first made using a serum broth which had been heat-sterilized. It gave irregular results, due to heat sterilization or to the action of the serum enzymes, especially on maltose and xylose.

Indol formation was tested on the basic broth medium after 4 or 5 days incubation. A slight modification of Kovacs' test (1928) was used to detect the formation of indol. It consisted of the addition of 1 cc. of ether to each tube, which concentrated the indol at the surface of the medium and allowed a rapid and definite purple indol-ring to form. Nitrate reduction tests were made on 5 day-old cultures in basic broth to which 0.2 per cent sodium nitrate had been added. Hydrogen sulphide and Voges-Proskauer tests were made with Kligler's lead acetate and 2 per cent glucose broth respectively, the latter tested by the addition of 50 per cent caustic potash and read after 24 hours.

Mouse pathogenesis was determined by the intraperitoneal injection of each organism into three mice. The first two in-

jections of 0.5 cc. each were made with 18- to 24-hour broth cultures, while the third test was done with 1 cc. of a dilute suspension of a young serum-agar culture. A series of mouse passages was also carried out with some of the strains to determine the effect of successive animal passages. The dead animals were autopsied and cultures made from the heart to diagnose the cause of death.

Agglutination technic

Twelve rabbits were injected, 25 times each during a period of 90 days, with 12 different broth antigens. The first 15 injections were made intravenously and intraperitoneally with heat-killed cultures, producing homologous titres lower than 1:1,600. In the remaining 10 inoculations live cultures were injected subcutaneously, intraperitoneally, and some intravenously. The final homologous titre of most sera was 1:32,000. There were, however, two strains of very poor antigenic ability which did not cause the production of such a high titre.

The agglutination tests were made with agar-growth antigens suspended in physiologic saline in two series of increasing serum dilutions. The first series consisted of dilutions ranging between 1:25 to 1:3,200 while the second series ranged from 1:250 to 1:32,000. The tests, with their controls, were then placed at a temperature of 40–43°C. and the readings taken after 72 and 96 hours of incubation.

EXPERIMENTAL RESULTS

Morphology and cultural characteristics (see table 1)

The organism proved to be so pleomorphic that it could not be typed on the basis of morphology, nor, at times, distinguished by this means from contaminating forms. Pleomorphicity was most noted in broth and carbohydrate media while uniformity in shape was increased by repeated transfers on solid-medium or animal passage. Carbohydrate media, especially when fermented, were responsible for the greatest pleomorphicity of the organism; there were, in some cases, organisms 3 to 4 times their

normal size as well as long chain formations. Capsule or pseudo-capsule formation was observed on some of the larger cells.

In serum-agar plates fluorescent, non-fluorescent, and intermediate colonies were distinguished. A differentiation in species could not be made on the basis of colony characteristics. The occurrence of fluorescence was not constant but fluctuating and was partially associated with continuous serum-agar transfers or with a series of mouse passages. The changes of colonies toward the fluorescent mucoid type occurred spontaneously in single colonies, but were usually short-lived. In general it was found that the intermediate fluorescent forms were the least stable, while the non-fluorescent forms were the most stable. Recently isolated strains showed the fluorescent characteristic in the majority of cases.

In broth, most of the organisms grew diffusely, but there were some strains that showed an early precipitation into granular or floccose particles, leaving the supernatant fluid clear or almost so. This latter characteristic was later correlated with the avirulence of the culture. A partial sedimentation of a mucoid-stringy nature was noted in a number of the strains. None of the strains were motile.

Two main divisions could be made on the basis of hemolysis. The hemolytic forms corresponded to Newsom and Cross' *Pasteurella hemolytica*, and the remaining strains corresponded to the typical *Pasteurella* group of the same authors.

Bile-solubility tests proved negative for all strains. No satisfactory species or group differentiation could be obtained from the study of dye bacteriostasis with six different dyes. There was, in general, however, a tendency of the avian strains to grow in higher concentrations of crystal violet. The growth-limits of the avian strains ranged between the dilutions of 1:15,000 to 1:25,000 whereas the other strains' growth-limits ranged between 1:33,000 to 1:50,000.

The pH range of the organisms in proteose-peptone broth as tested with brom-thymol blue, chlorphenol red, and phenol red was between 6 and higher than 8.5 with an optimal pH between 7.2 and 7.4.

TABLE 1

Biochemic, cultural and biologic characteristics of 44 representative Pasteurella organisms

Strains	Host Origin	Xylose	Arabinose	Dulcitol	Glycerol	Adonitol	Mannitol	Sorbitol	Inositol	Lactose	Maltose	Sucrose	Trehalose	Raffinose	Hemolysis	Indol prod.	Nitrate red.	Bile solubil.	Mouse pathogenesis	Colony fluorescence	Broth characteristics	Morphology
		Pentoses		Alcohols						Di- and Tri-saccharides												
Group I																						
104	Avian	−	+	+	±	−		+	−	−	−	+	−	−		±	+	−	−	N	D	R
106	Avian	−	+	+	−	−		+	−	−	−	+	−	−	−	+	+	−	+++	F	M	R
335	Avian	−	+	−	+	−		+	−	−	−	+	−	−	−	±	±	−	+++	F	D	I
1525	Avian	−	+	+	+	−		+	+	−	−	+	−	−	−	+	+	−	+++	F	D	R
T	Avian	−	+	+				+	−		−	+			−	+	+	−		F	D	R
57	Bovine	−	+	+	+	−	+	−		−	−	+	−	−	−	+	+	−	+++	F	D	R
226	Bovine	−	+	+	+	−		+	−	−	−	+	−	−	−	+	+	−	++	F	D	I
31	Ovine	−	+	+	+	−		+	+	−	−	+	−	−	−	+	+	−	+++	F	D	R
642	Ovine	−	+	+	+	−		+	+	−	−	+	−	−	−	+	+	−	++	N	D	I
150	Porcine	−	+	+	−	−		+	+	−	−	+	−	−	−	+	+	−	+++	F	D	R
236	Porcine	−	+	+	±	−		+	−	−	−	+	−	−	−	+	+	−	++	N	M	I
257	Equine	−	+	+	−	−		+	−	−	±	+	−	−	−	+	+	−	+	N	D	I
Group II																						
120	Equine	+	−	−	−	−	±	−		−	−	+	−	−	−	+	+	−	+++	N	M	I
243	Equine	+	−	−	+	−		+	−	−	−	+	−	−	−	+	+	−	+	N	D	I
84	Bovine	+	−	−	−	−		+	−	−	−	+	−	−	−	+	+	−	++	N	D	R
109	Bovine	+	−	−	−	−		+	−	−	−	+	−	−	−	+	+	−	++	N	M	R
153	Bovine	+	−	−	+	−		+	−	−	−	+	±	−	−	+	+	−	++	F	D	R
1932	Bovine	+	−	−	−	+		+	−	−	−	+	−	−	−	+	+	−	+++	F	D	I
116	Buffalo	+	−	−	−	−		+	−	−	−	+	−	−	−	+	+	−	++	N	M	I
779	Buffalo	+	−	−	+	−	+	+		−	−	+	−	−	−	+	+	−	+++	N	M	I
4300	Buffalo	+	−	−	−	+		+		−	−	+	−	−	−	+	+	−	+++	N	M	I
168	Ovine	+	−	−	+	−		+	−	−	−	+	−	−	−	+	+	−	+++	F	D	R
169	Ovine	+	−	−	+	−		+	−	−	−	+	−	−	−	+	+	−	+++	F	M	R
175	Ovine	+	−	−	−	−		+	−	−	−	+	−	−	−	+	+	−	++	F	D	R
134	Porcine	+	−	−	−	−		+	−	−	−	+	−	−	−	+	+	−	+++	F	M	I
70	Porcine	+	−	−	−	N		+	−	−	−	+	−	−	−	+	+	−	+++	N	D	I
70R	Porcine	+	−	−	−	−		+	−	−	−	+	−	−	−	+	+	−	−	N	M	R
138	Rabbit	+	−	−	−	−		−	−	−	−	+	−	−	−	+	+	−	+++	N	D	R

Mouse Pathogenesis: (+++) killing in 6–9 hours; (++) in 10–15 hours; (+) in 16 or more hours.

Fluorescence: (F) fluorescent; (N) not fluorescent.

Carbohydrates: (+) fermented; (±) slight fermentation; (−) not fermented. Broth characteristics: (D) diffuse; (M) mucoid precipitate; (P) complete precipitation.

Morphology: (R) regular; (I) irregular.

(*) Doubtful classification.

TABLE 1—*Concluded*

STRAINS	HOST ORIGIN	Xylose	Arabinose	Dulcitol	Glycerol	Adonitol	Mannitol	Sorbitol	Inositol	Lactose	Maltose	Sucrose	Trehalose	Raffinose	HEMOLYSIS	INDOL PROD.	NITRATE RED	BILE SOLUBIL.	MOUSE PATHOGENESIS	COLONY FLUORESCENCE	BROTH CHARACTERISTICS	MORPHOLOGY
		PENTOSES		ALCOHOLS						DI- AND TRISACCHARIDES												
Group III																						
95*	Ovine	−	+	+	±	−		+		−	±	+	−	−	−	+	+	−	−	N	D	R
130	Ovine	+	+	+	−	−		+		−	−	+	−	−	−	+	+	−	++	F	M	R
412	Ovine	+	+	+	+	−	+	+		−	−	−	+	−	−	+	+	−	++	F	D	R
R	Bovine																		++	F	D	R
Group IV—Pasteurella hemolytica																						
161	Ovine	−	−	−	−	−			−	+	+	+	+	+	+	+	−	−	−	N	M	R
164	Ovine	+	−	−	+	−		+	+	+	+	+	−	−			+	−	−	F	M	I
884	Ovine	±	±	±	+			+		+	±	+	+				−		+	F	D	I
159	Bovine	+	±	±	+	−		+	+	±	+	+	−	+	+	−	+	−	−	N	P	I
165	Bovine	+	±	±	+	−		+	+	+	+	+	−	+	+	−	+	−	−	N	P	R
178	Bovine	+	−	−	+	−		+	+	+	+	+	+	+	−	−	±	−	++	F	D	I
Variants																						
35M	Porcine	+	−	−						−	−	+	−		−	+		−	+++	F	D	R
217M	Bovine	−	−	−	−	−		±	−	−	−	+	−	−		+	+	−	−	F	M	I
590M	Bovine	+	−	−	+	−		+		−	−	+	+	−	−	+	+	−	+	F	P	R
33R	Ovine	+	−	−	+	−		+		−	−	+	−	−	−	+	+	−	−	N	P	R
234R	Ovine	±	−	±	±	−		+		−	−	+	±	−	−	+	+	−	−	N	P	I
242R	Buffalo	+	−	−	−	−		±		−	−	+	−	−	−	+	+	−	−	N	P	R
122R	Feline	+	−	−	−	−		±		−	−	+	−	−	−	+	+	−	−	N	P	I

Biochemic and pathogenesis characteristics (see table 1)

The fermentation study of 22 carbohydrates, alcohols and glucosides revealed that xylose, arabinose, and dulcitol were sufficient for the classification of the typical non-hemolytic strains into two main groups. The atypical hemolytic forms could be separated from the rest by their ability to ferment inositol, lactose, maltose, trehalose, and raffinose. This group, which corresponded to Jones' type I and Newsom and Cross' *Pasteurella hemolytica* was classified as group IV. The other carbohydrates and alcohols were carried as contamination controls. The three main carbohydrates were tested a total of 3 to 8 times per culture depending on the strain in use. The results obtained warrant

the sub-division of the typical forms into two main groups: Group I ferments arabinose and dulcitol but not xylose, which corresponds to Khalifa's type A; group II ferments xylose but not arabinose nor dulcitol, which corresponds to Khalifa's type C. Group III included three strains which fermented all three carbo-hydrates and an additional strain, 95, of doubtful classification, but which showed the fermentation characteristics of group I.

In addition to the carbohydrates and alcohols reported in table 1, the hexose carbohydrates glucose, levulose, galactose, and mannose were fermented by all the strains. The pentose rhamnose, the glucoside salicin, and the polysaccharides inulin, starch, and dextrin were not fermented by any of the typical strains.

The fermentation of xylose occurred somewhat slowly, while that of arabinose occurred more rapidly. A uniformity in the fermentation of arabinose and dulcitol was observed in almost all of the strains. Dulcitol showed somewhat variable results. It apparently inhibited the growth of the organisms, due, possibly, to heat sterilization and prolonged incubation before inoculation.

Four strains, group I cultures 232 and 236, and group II cultures 84 and 1932, which were studied through a period of two years, showed changes in the fermentation of xylose, arabinose, and dulcitol. These organisms were tested a total of 4 to 8 times and the results were quite surprising (see table 2), as they alternated through the experiment from the characteristics of groups I to II.

The variations in fermentation of arabinose and xylose were adequately controlled by comparisons with the other normally-acting cultures and by the constant check on such contaminant-control carbohydrates as lactose, maltose, and sucrose, as well as by additional control tests with raffinose, trehalose, rhamnose, inositol, indol production, colony characteristics, mouse patho-genesis, and dye bacteriostasis. Further evidence that the biochemic variability was not caused by contamination was provided by the constant uniformity in the fermentation of dulcitol and arabinose, which, at the same time, provided evidence that the chemical nature of the carbohydrates had not changed.

Mouse pathogenesis was of little value in the separation of the strains into groups or species.   The strains belonging to group IV and those of the variant group were either entirely apathogenic or of low virulence.

In general, no relation could be found between colony characteristics and virulence.   On the other hand, when four serial mouse passages were carried out on a bovine strain, 1932, and an ovine strain, 31, there was an increase in colony fluorescence and in virulence to the extent that they caused the death of two chickens.

TABLE 2

Biochemic fermentation variations

STRAINS		1	2	3	4	5	6	7	8	9	10	ORIGINAL CARBOHYDRATE GROUPS	FINAL CARBOHYDRATE GROUPS
Bovine	Arabinose		0	0					+	+		II	I
232	Xylose		+	+					0	0			
Porcine	Arabinose		0	0					+	+	+	II	I
236	Xylose		+	+					0	0	0		
Bovine	Arabinose	0	0	+	+				+	±	0	II	II
84	Xylose		+	0	0				0	+	+		
Bovine	Arabinose		0	0		+	+	+	+	±	0	II	II
1932	Xylose		+	+		0	0	0	+	+	+		
Dates of tests......		2/35	5/35	7/35	9/35	10/35	11/35	12/35	7/36	8/36	1/37	1/37	1/37

(+) = fermentation in less than 8 days; (±) = fermentation in more than 8 days; (0) = no fermentation.

There were marked stable variations in virulence in some individual strains.   Strain 642 was completely avirulent and strain 226 was apathogenic in a number of trials, but both strains gradually became more and more virulent.   The reverse was true with strains 217 and 259.

Serologic characteristics

Some of the strains revealed an auto-agglutinating tendency. This occurred for the most part in strains which showed poor growth on common agar and in strains showing early broth pre-

TABLE 3

Classification of 44 representative Pasteurellae on the basis of selective agglutinations

STRAINS	HOST ORIGIN	GROUP I					GROUP II			GROUP III		VARIANTS		RABBIT NORMAL	BOVINE SERUM
		Av. 100	Bv. 57	Bv. 220	Su. 150	Eq. 259	Su. 70	Ov. 108	Bf. 770	Ov. 05	Ov. 412	Ov. 234	Su. 35M		

Group I

STRAINS	HOST ORIGIN	Av. 100	Bv. 57	Bv. 220	Su. 150	Eq. 259	Su. 70	Ov. 108	Bf. 770	Ov. 05	Ov. 412	Ov. 234	Su. 35M	RABBIT NORMAL	BOVINE SERUM
104	Avian	+++	+++	+++	+++	+++	+	+++	+++	+++	++	++	+	++	+
106	Avian	+++	+++	+++	+++	+++	+	+++	+++	+++	++	+	+	+	+
335	Avian	++	+++	+++	+++	+++	+	+++	+++	++	+	−	+	−	+
1525	Avian	++	+++	+++	+++	+++	+	+++	+++	+++	+	+++	+++	++	+
T	Avian	++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+	++	+
57	Bovine	++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	−	+	+
226	Bovine	++	+++	+++	+++	+++	+	+++	+++	+++	+	−	−	+	+
31	Ovine	++	+++	+++	+++	+++	−	+++	+++	+++	−	−	−	−	+
642	Ovine	++	+++	+++	+++	+++	−	+++	+++	+++	−	−	−	−	+
150	Porcine	++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	++	+
236	Porcine	++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	++	+
257	Equine	+	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	++	++

Group II

STRAINS	HOST ORIGIN	Av. 100	Bv. 57	Bv. 220	Su. 150	Eq. 259	Su. 70	Ov. 108	Bf. 770	Ov. 05	Ov. 412	Ov. 234	Su. 35M	RABBIT NORMAL	BOVINE SERUM
120	Equine	++	+++	+++	+++	+++	+++	+++	+++	+	++	+++	++	++	++
243	Equine	−	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
84	Bovine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	−	++
109	Bovine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	−	++
153	Bovine	++	+++	+++	+++	+++	+++	+++	+++	−	+++	+++	+	−	+
1032	Bovine	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	++
116	Buffalo	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
779	Buffalo	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	+++	+++	++
4300	Buffalo	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	++
108	Ovine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+++
169	Ovine	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	−	−	++
175	Ovine	+++	+++	+++	−	++	+++	+++	+++	+	+++	+	+	−	++
134	Porcine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
70	Porcine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
70R	Porcine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
138	Rabbit	+++	+++	+++	++	+++	+	+++	+++	+++	+++	+++	+++	+++	++

Group III

Strain	Source												
95*	Ovine	+	−	−	−	−	+⁺	+	−	+	+	+	+
130	Ovine	+	−	−	+	⁺⁺⁺	+	−	+	+	+	+	+
412	Ovine	+	−	+	+	⁺⁺⁺	+⁺	+	+	−	+	−	+
R	Bovine	−	−	−	−	+⁺	+⁺	−	⁺⁺	−	+	+	+

Group IV—Pasteurella hemolytica

Strain	Source												
161	Ovine	−	−	−	−	+	+	+	+	−	−	+⁺	+
164	Ovine	+	+	+	+	+	+	+	+	⁺⁺	+	+	+⁺
884	Ovine	−	−	−	−	+	−	+	+	−	−	+⁺	+
159	Bovine	+	+	+	+	+	+	+	+	+	+	+	+
165	Bovine	+	+	+	+	+	−	+	+	+	+	+	⁺⁺
178	Bovine	+	+	−	+	+	+	+	−	+	+⁺	+	+

Variants

Strain	Source												
35M	Porcine	+	+	−	+	+	−	⁺⁺	+⁺	−	⁺⁺	−	+
217M	Bovine	+	+	+	+	+	+	+	+⁺	+	+	⁺⁺	+
590M	Bovine	+	+	+	+	+	+	+	+	+	+	+	+
33R	Ovine	+	+	+	−	+	−	+	+	+	+	−	+
234R	Ovine	⁺⁺	+	+	+	⁺⁺	+⁺	⁺⁺	+	⁺⁺	+	−	+
122R	Feline	+	+	+	+	+	+	+	+	+	⁺⁺	+	+

(+) = 1:50–1,600; (++) = 1:2,000–8,000; (+++) = 1:16,000–32,000.
(Av.) = aviseptica serum; (Bv.) = boviseptica serum; (Su.) = suiseptica serum; (Ov.) = oviseptica serum; (Eq.) = equiseptica serum; (Bf.) = bubaliseptica serum. (*) = doubtful classification.

cipitation. To decrease this effect, the antigens of these strains were prepared with a lower salt concentration, and the tests were read at more frequent intervals.

By using ten immune sera it was possible to classify 44 *Pasteurella* strains into 4 groups (see table 3) by means of the agglutination test. For that purpose 5 group I sera, 3 group II sera, and 2 group III sera were used. The groups observed corresponded to the carbohydrate grouping mentioned previously.

Group I strains gave the highest agglutination titres with 5 group I sera. Some of the strains were agglutinated by 2 group II sera and especially by one which was considered of intermediate character. These strains were also agglutinated by 1 group III serum, 95, which was the least typical of the group.

Group II organisms were more specific in their agglutination reactions, since they were agglutinated in the higher titres only by the group II sera. Individual agglutination variations were encountered in this group more often than with group I strains. Certain of the equine strains showed a tendency to agglutinate at high titres with group III serum 95. Some of the bovine and ovine strains tended to agglutinate at higher titres with group I serum 259, while porcine strain 134 agglutinated to a moderate titre with group I strain 57, which was otherwise specific to group I. The rabbit strains appeared to vary most from the typical form; they acted as intermediates to group III.

Group III includes 3 organisms of miscellaneous characteristics which gave a high agglutination titre with serum 412. Strain 95 was homologous in agglutination characteristics and acted otherwise as an intermediate variant of group I.

Group IV was composed of serologic strains bearing no relation to any of the sera. The organisms of this group corresponded to Newsom and Cross' *Pasteurella hemolytica* species, for which, unfortunately, no homologous serum was available.

The variant group which was set apart because of its cultural characteristics was shown to contain organisms of low agglutinability and poor antigenicity. Sera 35M and 234R reached the final homologous agglutination titre of 1:2,000 and 1:800 respectively, even though culture 35M was very pathogenic.

Two control sera were used. One was from a normal rabbit of the same origin as most of the rabbits used during the experiment. Another was from a rabbit immunized with *Brucella abortus*. Both these sera showed agglutination titres of certain strains ranging from 0 to 1:800. The animals showed no history of a previous infection so that all the agglutinations under 1:1,600 were considered as insignificant in the interpretation of agglutination results. Since the results of both sera were comparable, only one is included in table 3.

The other serum was a composite *Brucella-abortus*-positive cow-serum. This serum showed fairly high titres with the group II organisms, as well as with two group IV and one group I equine strain.

Cross-agglutination tests on the rabbit sera with a polyvalent *Brucella abortus* antigen were negative. Similarly, tests were made with all the sera on 6 *Salmonella* and 2 *Escherichia* strains with negative results, even though some of the agglutination titres were as high as 1:200, which was attributed to an acquired immunity of the rabbits tested.

Variability

During the course of the study of 114 *Pasteurella* cultures, eight strains were found which were considered to be variants on the basis of a granular or mucoid precipitate in broth cultures, lack of virulence for mice and poor agglutinability with immune sera.

Of these organisms, strain 33R, was a permanent "R" form. Its "S" pathogenic form was available as culture 169, which was provided by Newsom. Both of these strains differed in every respect except their carbohydrate fermentation behavior.

Strain 35M,[2] on the other hand, behaved as a pathogenic

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[2] Culture 35M appeared similar to the virulent capsulated forms described by Priestley (Brit. Jour. Exp. Path., 17: 374–378, 1936). The behaviour of this culture as well as some of the other recently isolated organisms of avian origin seemed to conform to this type. According to the above author, the poor antigenicity and agglutinability of such strains are due to the presence of a capsule which is present when the organism is virulent and recently isolated.

and poorly antigenic mucoid "M" form originating from a recent Argentine field case isolation.

The other variants included strains 234R, 112R, and 242R, as well as strains 217M and 590M. These cultures presented varying degrees of irregularity in carbohydrate fermentation and agglutination behavior, which were attributed to their different degrees in phase changes. These conclusions were based upon colony and broth characteristics, and mouse pathogenesis. These organisms represented possible "R", "M" and intermediate forms.

An additional variant studied included strain 70R which was isolated from "S" to form 70. This culture differed from its original culture in rough colony characteristics, precipitation in broth, and lack of mouse pathogenesis. Yet, it was similar serologically and biochemically, and it reverted once after the second month of isolation so that it probably represented an intermediate of the type "SR".

DISCUSSION

The purpose of this investigation was to correlate all the available literature on the inter-relationship of the hemorrhagic septicemia Pasteurellae and to test the validity of the zoologic classification and improve upon the various typing techniques which have been reported.

The biochemic, cultural, biologic, and serologic results revealed that the Pasteurellae were composed of widely differing strains of organisms which warranted their classification into two separate species, as suggested by Newsom and Cross. These authors, in 1932, gave the species name *Pasteurella hemolytica* to an atypical, avirulent, hemolytic, and non-indol-forming group composed of strains of bovine and ovine origin. The second group, which they termed the typical group, was of great homogeneity and included strains from avian, bovine, ovine, porcine, equine, buffalo, rabbit, cat, mink, and deer origin. The origin of these strains, however, could not be detected experimentally by either morphologic, cultural, biochemic, or serologic methods.

The main argument for the host-specificity classification of the group up to now has been epidemiologic. Still, this argument has never been adequately nor sufficiently sustained either epidemiologically or experimentally. On the other hand, many epidemiologic reports, cross-pathogenesis studies and cross-immunity tests support the inter-relation of the various species. Of importance are the reports of Baumgarten and Migge, who have gone so far as to state, that epidemiologically the Pasteurellae, with their various changes and types, follow a definite cyclic development in their adaptation to the various hosts. Similar conclusions are encountered throughout the literature. Most of these reports emphasize that cross-pathogenicity is a factor related to differential virulence and host adaptation.

On the basis of the bacteriologic results obtained, on the well-grounded historical epidemiologic data of the species unity, as well as the similar pathology of the disease in various animals, it is possible to group all the typical Pasteurellae in a single species possessing the characteristics given by Lignières (1900), Vorloud (1908), and Manninger (1934). In conformity with these facts, the name *Pasteurella multocida*, Kitt, 1885, *n. comb.* is suggested to include all of the typical, indol-producing, non-hemolytic hemorrhagic septicemia organisms. The name originates from the first one given to an organism involving more than one host. It is also one of the first names given to the microörganism, and though originally a bi-nomial, it is the most suitable. This grouping is not the first one, since Hueppe, in 1886 (cited by Tonaka, 1926), and Hutyra (1928), used single species names, also bi-nomial in character, to identify the organism.

The typical forms were consistently subdivided by their agglutination and carbohydrate fermentation tests into two main groups, and possibly a smaller, third group, all of which definitely crossed the host species lines. These groups differed in the fermentation of xylose, arabinose, and dulcitol and in agglutination reactions.

Group I contained all of the avian strains as well as representative strains of various other hosts. This group was highly

homogeneous, containing a predominance of the organisms with a fluorescent type of colony, a stable, diffuse appearance in broth, and a tendency toward a regular cell morphology.

Group II contained strains of all origins except avian. It could be subdivided serologically into three or four subgroups depending on the degree of relationship to the members of group I. Strain 70 was possibly the true exponent of the group, while strain 168 was quite closely related to group I. The intermediate character of many of the strains of this group was revealed by the predominance of non-fluorescence, mucoid appearance in broth, and tendency toward an irregular cell morphology.

Group III strains did not show a uniform fermentation of the basic carbohydrates. Three of the strains fermented xylose, arabinose, and dulcitol. The organisms showed only a high-titre homologous agglutination and they were devoid of mouse pathogenicity; they possibly represented intermediate forms between groups I and II. Most of the strains possessed fluorescent colony characteristics, a diffuse broth growth, and a regular cell morphology. It may be of speculative interest to note that the apparent grouping is based only upon the characteristics which the various strains revealed at the time they were studied, and are representative of cyclic changes which the organism passes through under different environments. These changes may be due to the acquisition or loss of certain antigenic fractions of the organism.

It was not possible to differentiate host species nor groups on the basis of dye bacteriostasis. Avian strains of both American and Argentine sources, which appeared to be the most homogeneous and stable, were the most resistant to crystal violet.

Bacterial variations, both of a temporary and a permanent nature, were observed. The former occurred as spontaneous, short-lived changes in fluorescence, often environmental in nature, or in mouse pathogenesis or carbohydrate fermentation, suggesting cyclic changes. The bacterial variations of permanent nature were classed as rough "R" and mucoid "M" forms. Intermediate forms were also encountered. All of these

types of variations have been described in the literature, but their interpretation was difficult, because adequate inter-relating studies were absent. It is believed, therefore, that a careful study of the cultural, morphologic, biochemic, and serologic variations would be of great value in interpreting the changes which the organism exhibits *in vitro* and *in vivo* through long periods of time.

CONCLUSIONS

1. Two distinct types of organisms were studied; they differed in cultural, biochemic, serologic and pathogenic characteristics. One type included typical strains usually associated with hemorrhagic septicemia; the other included atypical forms designated as *Pasteurella hemolytica* by Newsom and Cross.

2. The typical strains were divided into two rather distinct subgroups, and a third, less distinct one, on the basis of xylose, arabinose and dulcitol fermentation and by agglutination reactions.

3. Evidence presented invalidates the present zoologic species classification. The name *Pasteurella multocida* Kitt, 1885, *n. comb.*, which includes all typical strains, is suggested to take the place of all the host species names, which are now in common use.

4. Two types of variability were encountered. The permanent variants were of "R" and virulent "M" types, with their intermediate forms. The temporary variants which were produced in many respects by environmental effects on cultural characteristics, mouse pathogenesis, and biochemic reactions, suggested possible cyclic changes in the organism; these changes may account for the variability of results obtained by many previous investigators.

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CHROMOGENIC STRAINS OF ESCHERICHIA[1]

RALPH P. TITTSLER

*Department of Bacteriology, University of Rochester, School of Medicine and
Dentistry, Rochester, New York*

Chromogenic strains of the genus *Escherichia*, obtained from
human feces and from water, have been described briefly in a
preliminary report (1937). Except for the production of a
"reddish-orange" pigment, these strains cannot be distinguished
from certain well-established non-chromogenic species of *Esch-
erichia*. On the basis of minor differences, they fall into three
types. They form gas, produce indol, yield negative Voges-
Proskauer reactions, and fail to liquefy gelatin. Thus, they are
distinctly different from most of the "yellow" pigment-forming
"colon" or "colon-like" organisms which have been isolated
from cereals, water, *etc.* by other workers, and which have been
mentioned by Parr (1937), and by Lehmann, Neumann and
Breed (1931). On the other hand, the present strains may be
identical with some of those encountered previously. This point
cannot be settled, because the descriptions which have been
recorded in the literature are incomplete, except for those of
Parr (1937). Two of the present strains seem to be identical
with the culture described by him.

The present paper records in detail the characteristics of
five strains of chromogenic coliform bacilli and compares them
with well-recognized species of *Escherichia*.

MATERIALS AND METHODS

Three of the bacterial strains were isolated in 1932 from eosin
methylene-blue agar during the course of routine bacteriological

examinations of feces and water, and were considered to be typical members of the genus *Escherichia* until they were cultivated on nutrient agar.   One strain was obtained in 1936 from the feces of an adult who had suffered for years from chronic constipation. On eosin methylene-blue agar and on Endo's medium, inoculated directly from a fecal suspension, it also appeared to be a typical member of the *Escherichia*, but on blood agar it was distinctly chromogenic.   Several isolations were made from each medium and each subculture proved to be chromogenic on nutrient agar. One strain was made available through the courtesy of Dr. R. S. Breed, who had received it from Dr. A. J. Kluyver of Delft, Holland.   Originally, it came from Dr. M. W. Beijerinck's collection and "had been carried in the Delft collection for a long time, possibly for as long as 20 years."   The original source is not known.

The media and methods employed to determine the various characteristics were the same as those described by Tittsler and Sandholzer (1935).   Hemolysis was determined in pork-infusion agar containing rabbits' blood, according to the method of Paulson and Brown (1931).

From 3 to 6 subcultures were isolated from each parent strain, and the cultural characteristics were determined for each subculture.   During the course of the present study all of the characteristics were redetermined at least once.

RESULTS

The results of a detailed study of the cultural characteristics of five strains of chromogenic coliform bacilli are recorded, for the most part in tables 1 and 2.   All of the strains were found to possess certain features in common.   These are shown in table 1. They fermented glucose and lactose with the production of acid and gas; failed to liquefy gelatin; produced indol; gave positive methyl-red tests; and yielded negative Voges-Proskauer reactions.   Carbohydrates were fermented rapidly, with the exception of salicin and glycerol, in which acid formation did not occur until the second or third day of incubation.

These cultures differed among themselves in certain char-

acteristics, as shown in table 2.   On the basis of these differences, the five strains fell into three types.   Strains 219 and 870, from water and from human feces respectively, were alike.   Strains 220 and 251 were alike and, evidently, they were identical with the culture described by Parr (1937).   Strain 221 differed from the others.

TABLE 1

Characteristics common to all five strains of chromogenic coliform bacilli

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS	CARBOHYDRATES FERMENTED WITH ACID AND GAS	CARBOHYDRATES NOT FERMENTED
Bacilli..............................	l-Arabinose	Sucrose
Non-sporogenous......................	d-Galactose	Cellobiose
Gram-negative........................	d-Glucose	Raffinose
Aerobic..............................	d-Levulose	a-Methylglucoside
Gelatin not liquefied..................	d-Mannose	Dulcitol
Indol produced	d-Xylose	l-Erythritol
Nitrates reduced......................	d-Lactose	l-Inositol
Methyl-red positive...................	Maltose	Inulin
Voges-Proskauer negative.............	Trehalose	Starch
H₂S not produced.....................	Glycerol	
Citrate not utilized...................	Mannitol	
Milk acidified, coagulated and reduced..	Sorbitol	

TABLE 2

Differential characteristics of five strains of chromogenic coliform bacilli

	221	220	251	219	870
Salicin.........	AG	AG	AG	—	—
Adonitol.......	AG	—	—	AG	AG
Hemolytic type	Beta	Gamma	Gamma	Beta	Beta
Motility........	+	+	+	—	—
Source.........	Feces	Feces	Unknown	Water	Feces

AG = Acid and gas.

The color of the pigment produced by the parent cultures was designated by the writer as "reddish-orange" (1937).   When classified according to Ridgway's Color Standards (1912), however, it is xanthine orange.   Parr characterized the pigment produced by his strain as "a distinct golden-brown" and "practically the same color as that observed for a typical *Staphylococcus*

aureus." When strains 219 and 221 were plated on nutrient agar, they usually yielded a few colonies which had very little color, but subcultures from such colonies formed as much pigment as did the parent strains. On the other hand, strains 220 and 870 yielded some colonies which were practically non-chromogenic and subcultures from them developed but little pigment. When these slightly chromogenic substrains were plated and isolations made from the least chromogenic colonies, cultures were obtained which develop no detectable pigment in colonies and very little in mass growths. Otherwise, the slightly chromogenic strains did not differ from the most chromogenic substrains of the same parent culture. All of the parent strains have retained their chromogenicity constantly during repeated cultivation on nutrient agar, for as long as six years.

DISCUSSION

The results of a detailed study of the cultural characteristics of five chromogenic strains of coliform bacilli show that they are more closely related to members of the genus *Escherichia* than to those of any other genus. On the basis of chromogenesis alone, they might be allocated to either of the genera, *Flavobacterium* or *Serratia*. This designation would seem to be illogical, however, because their failure to liquefy gelatin, their fermentation of numerous carbohydrates with the formation of gas, and their production of indol, are different from those recorded for members of either of these genera. When the characteristics of the present strains are compared in detail with those recorded in Bergey's Manual (1934) for certain members of the genus *Escherichia*, one finds a close agreement, except for the production of pigment. Thus, strains 219 and 870 differ from *Escherichia acidilactici*, and strains 220 and 251 from *Escherichia paragruenthali*, only in their inability to ferment raffinose. Strain 221 differs from *Escherichia paragruenthali* in its ability to ferment adonitol and in its inability to ferment raffinose. The writer, however, has found that raffinose is not fermented by members of the *Escherichia* genus which do not ferment sucrose, *i.e.*, not by members of the coli-communis group. Thus,

except for chromogenesis, strains 220, 221 and 251 would be classified as *Escherichia paragruenthali* and strains 219 and 870 as *Escherichia acidilactici*. Strains 220 and 251 are identical with the strain described by Parr (1937) and for which he proposed the name *Bacterium aurescens, n.s.,* because it differed from *Escherichia paragruenthali* "not only in being chromogenic but in failing to ferment raffinose." Whether this proposal be acceptable or not depends upon the point of view. If strains like 220 and 251 are given a specific name, the argument can be raised that strains 219 and 870, and 221 deserve similar consideration on the basis of differences in the fermentation of salicin and adonitol and in hemolysis. The writer does not favor the segregation of closely related strains into new species, unless the procedure serves a useful purpose or cannot be avoided. Accordingly, no additional specific names are proposed for the present chromogenic strains. It seems sufficient to consider them as chromogenic varieties of either *Escherichia paragruenthali* or *Escherichia acidilactici,* depending upon their fermentation of salicin.

SUMMARY

A detailed study of the cultural characteristics of five strains of chromogenic coliform bacilli, isolated from water and human feces, is presented. On the basis of differences in the fermentation of salicin and adonitol and in hemolysis, these strains fall into three distinct types. Attention is directed to the relationship of these strains to *Escherichia paragruenthali* and *Escherichia acidilactici,* and the opinion is expressed that they can conviently be considered as chromogenic varieties of these recognized species, thus avoiding the necessity for the creation of new species.

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STUDIES OF FRESHWATER BACTERIA

V. The Distribution of Siderocapsa Treubii in, Some Lakes and Streams[1]

YVETTE HARDMAN AND ARTHUR T. HENRICI

*Department of Bacteriology, University of Minnesota, Minneapolis, Minnesota,
and the Wisconsin Geological and Natural History Survey,
Madison, Wisconsin*

The genus *Siderocapsa* was created by Molisch in 1909 to include certain bacteria of striking morphology, found in fresh waters. Two species were described, *Siderocapsa treubii* (Molisch 1909) and *Siderocapsa major* (Molisch 1910). The former was found growing as a periphytic organism upon the leaves of submerged plants. It was described as coccoid, from 0.4μ to 0.6μ, occurring in colonies of not more than 8 cells, included in colorless, apparently mucoid material, the latter surrounded by a heavy irregular deposit of iron. Molisch described the cells as unstainable with the usual dyes, but staining red with Schiff's reagent for aldehydes. *Siderocapsa major* was found in the thin scum floating on the surface of stagnant pools. It differed from the preceding species in the somewhat elongated form of the cells, up to 1.8μ in length; the mucoid material was not so sharply delineated from the iron, and the colonies were considerably larger, up to 100 or more cells.

Molisch's observations apparently remained unconfirmed for some years. Cholodny (1926) doubted the existence of these forms as iron-depositing bacteria. He pointed out that ferric hydroxide is deposited upon the leaves of submerged plants independent of any bacterial activity, and also appears in the water scum, and that bacteria are found in the same places. He

looked upon the organisms described by Molisch as an accidental association of various bacteria with independently deposited iron.

Naumann (1928), however, observed the growth of these bacteria upon glass slides submerged in various waters, and confirmed Molisch's description. Naumann also observed another species, *Siderocapsa monoica*, occurring as single cells surrounded by an iron deposit. This species was found only once, growing upon the leaves of *Potamogeton natans*. On the basis of this single observation he divided the genus into two sections: *Monosiderocapsa* to include *S. monoica*, and *Polysiderocapsa* to include the two species named by Molisch.

A fourth species, *S. coronata*, was described by Redinger (1931). This is a plankton organism, i.e., it floats free in the water unattached to any surface. He points out that this habit of growth invalidates Cholodny's argument, since no plant material other than the bacteria is involved. It was found in Upper Lake Lunz, in Austria, and appeared in large foamy masses, at times as large as a fist. These zoogloeae were composed of coccoid bacteria, about 1μ in diameter, occurring in groups of 2 to 8 cells surrounded by mucoid material upon which iron and manganese were deposited in large quantities. Ruttner (1937) reported further on the distribution of this organism, which was found to be related to the oxygen stratification in various Alpine lakes. It was found most frequently at depths from 17 to 20 meters, where the oxygen range was from 0.12 to 0.30 mgm. per liter. The highest oxygen tension at which it was found was 4.66 mgm. per liter.

Dorff (1934), working with Naumann, reobserved *S. treubii* and *S. major*. The former species was found to stain in varying degrees with basic aniline dyes, although warming the stain was necessary in some cases. *S. major* was found not only in the scum on stagnant pools, but also upon the leaves of submerged plants. Both species were found only in still waters.

We have observed an organism presenting the characters of the genus *Siderocapsa* as described by Molisch, on slides which have been suspended in the waters of various lakes and streams. In many instances the iron deposit surrounding the colonies of

this organisms was the only iron deposit upon the slides. We believe, therefore, that this iron is deposited by the activities of the bacteria in question, and is not a mere accidental occurrence as proposed by Cholodny. We have found, contrary to the observation of Dorff, that these bacteria may grow abundantly on slides submerged in very rapidly flowing streams.

We have found upon our slides types of colonies which correspond in part with the two species described by Molisch. The first type has coccoid cells, 0.6μ to 1.6μ in diameter. From one to 30 cells are found in a colony, most frequently 6 to 8. The cells tend to occur in pairs, and stain deeply with Hucker's ammonium-oxalate crystal-violet solution. The outer edge of the mucoid capsule is well defined; it is round to elliptical, and may be from 2μ to 8μ across, most often about 4μ. Several colonies may be surrounded by a single continuous deposit of iron. This organism fits, in most particulars, Molisch's description of *S. treubii*.

The second type, far more common, is a small rod, 0.4 to 1.0μ in diameter and 2μ in length. The cells often stain unevenly. The colonies are usually elliptical in form, the mucoid capsule averaging about 2μ by 4.5μ. From 2 to 30 cells may be found in a colony, usually 4 to 6. Although the cells are rod-shaped, the other characteristics of this organism fit Molisch's description of *S. treubii* as well as those of *S. major*.

In all colonies the outer deposit surrounding the mucoid capsule, whether light or heavy, is rust brown when unstained, and is probably composed entirely of iron oxide. When the slides are stained with potassium ferrocyanide in dilute hydrochloric acid (the Berlin blue test for ferric iron), and counterstained with erythrosin, the cells are pink, the capsular material clear, and the whole outer structure blue. An attempt to stain one slide with Schiff's reagent failed. The slide was, however, several years old.

Although we have found on our slides forms corresponding fairly well with both *S. treubii* and *S. major*, we have also found, even on the same slide, so many intergrading forms, that we seriously question whether a differentiation of these two species

can be justified. It was observed that, when the colonies occurred in clusters, the individual colonies within a cluster were all of a single type as regards size and form of the cells. But the finding of so many doubtful intermediary forms prevented any recording of the two "species" separately. We shall refer to the organisms we have observed as *Siderocapsa treubii*, with the understanding that by this name we mean both types described by Molisch as well as the numerous intermediate forms.

The morphology of this organism as it occurs in its natural habitat is so striking that it can readily be recognized. The iron-encrusted colonies may be readily found with the low-power lens of the microscope. It is therefore an ideal organism for an ecological study by the direct microscopic method. We have searched for *Siderocapsa treubii* on nearly 2000 slides which have been suspended in the waters of 12 lakes and two streams in Minnesota and Wisconsin. These waters have varied widely in their physical and chemical characteristics, and it has therefore been possible to determine something about the environmental factors which determine the distribution of the microbe.

Although there was obviously a variation in the number of colonies on different slides no attempt has been made to count them. The organism has merely been recorded as "present" or "absent." In all instances several observations have been made at different times. All observations have been made during a period of the year when there was no ice in the lakes, with the exception of Lake Mendota, where observations were also made during the winter. In each lake, slides were submerged near the deepest part of the lake, being suspended at intervals from top to bottom. In the case of Lake Mendota and Lake Alexander, slides were also placed at several stations near the shore.

The occurrence of *Siderocapsa* in the various lakes and streams together with certain physical and chemical data regarding the lakes are presented in table 1. The chemical data have been obtained from various publications of Birge and Juday and their associates from the Wisconsin Geological and Natural History Survey. The chemical analyses recorded have been made on

surface samples. In those lakes where *Siderocapsa* is recorded as present, it was found at all depths, excepting the two deep, thermally-stratified lakes, Trout and Mendota.

In Trout Lake the organism was found in increasing abundance from the surface to a depth of 8 meters, below which level it was completely absent. The thermocline in this lake is found at about 12 meters, at which level the dissolved oxygen, tempera-

TABLE 1

Occurrence of Siderocapsa treubii in different types of lakes

LAKE	TYPE	OCCURRENCE OF SIDEROCAPSA	pH	BOUND CO_2	TOTAL Fe	ORGANIC C
				mgm. per liter	*mgm. per liter*	*mgm. per liter*
Forestry Bog..........	Seepage	Absent	5.2	1.0	0.32	12.3
Helmet................	Seepage	Absent	5.7	2.0	0.59	25.3
Weber.................	Seepage	Absent	6.0	1.5	0.03	2.8
Crystal...............	Seepage	Absent	6.0	1.5	0.07	1.6
Mary.................	Seepage	Absent	6.0	2.5	0.06	17.4
Muskellunge...........	Seepage	Absent	7.0	9.5	0.03	6.4
Boulder...............	Drainage	Present	7.5	13.6	0.48	7.7
Manitowish River*.....		Present				
Trout.................	Drainage	Present	7.5	19.0	0.03	3.7
Brazell	Drainage	Present	8.0	14.0	1.70	25.7
Little John...........	Drainage	Present	8.4	16.0	0.23	4.9
Allequash Creek†......		Present	8.6	16.6	0.32	6.7
Mendota..............	Drainage	Present	8.7	34 to 70	0.20	7.1
Alexander	Drainage	Present	Alkaline	Hard		

* Manitowish River empties into Boulder Lake; presumably the water is similar in composition.

† Allequash Creek drains Allequash Lake. Chemical analyses have been made upon the surface water of the lake.

ture and pH all decline rapidly. In Lake Mendota *Siderocapsa* was found only rarely, and its distribution was quite erratic. It was found once at a depth of 9 meters in August, but did not occur on any other slides submerged during the summer, either in deep water or near the shore. During the winter and spring, however, it was found abundantly at a depth of 18 meters, i.e., 2 meters above the bottom, and absent at all higher levels, save for single recordings at 15 meters and 12 meters.

Lake Alexander has been referred to in previous papers of this series as a seepage lake, and this is true at the present time. But until about ten years ago it drained into the Mississippi, and it presents the characteristics of a drainage lake. While, unfortunately, no chemical analyses are available, its water is obviously very hard and alkaline. *Siderocapsa* has been found on nearly all of the many slides which have been submerged in this lake, but is clearly much more abundant in the shallow weed-choked bays than in the open lake. While it has been found at all depths, it is more abundant on slides about 1 meter above the bottom than at other levels. Lake Alexander is not stratified.

The slides placed in the Manitowish River and Allequash Creek were stationed at places where the current flow was very rapid. In Allequash Creek slides were placed in a rapid where the current flow was so great that some of them were torn loose from their attachment. Nevertheless, these slides developed a heavy coating of bacteria in a few days, including abundant specimens of *Siderocapsa*.

The data presented in table 1 show clearly that *Siderocapsa treubii* occurs characteristically in alkaline, hard water lakes of the drainage type, and is absent in neutral or acid, soft water lakes of the seepage type. The distribution is clearly not determined either by the organic matter or by the abundance of iron in the water, although possibly on a quantitative basis correlations with these characters could be established. The amount of iron cannot, however, be the sole determining factor. Slides from Forestry Bog and Helmet Lake, with high iron contents, showed an abundance of other types of iron-depositing bacteria, especially *Leptothrix crassa* and related forms, but *Siderocapsa* was completely absent. Conversely, the latter microbe was fairly abundant at the surface of Trout Lake, with a very low iron content (0.03 mgm. per liter), and absent at the bottom, where there was much more iron (0.35 mgm. per liter) but a lower pH.

Of the various environmental factors considered, the pH of the water appears to be most important. Since *Siderocapsa treubii* is found only in alkaline waters, it seems highly probable

that it deposits iron by the oxidation of organic compounds, so-called "humates" of iron, the reaction probably occurring at the surface of the capsule. It has been shown by Halvorson and Starkey (1927) that truly autotrophic iron bacteria, i.e., those obtaining energy from the oxidation of inorganic ferrous to ferric compounds, could grow only in waters acid in reaction, since only in such waters could ferrous compounds remain in solution; and Turowska (1930) has shown that such organisms as *Leptothrix ochracea* and *Gallionella ferruginea* are rather sharply limited to waters neutral or acid in reaction. It is possible that the iron oxide is precipitated in the water spontaneously and merely adsorbed onto the capsule of *Siderocapsa*. This appears to be improbable because, as was mentioned, in many cases the only iron found on the slides was that deposited about the colonies of this microbe, although other types of bacteria or algae with mucoid capsules were frequently found. We therefore are of the opinion that *Siderocapsa treubii* is a heterotrophic bacterium which utilizes the organic radicle of organic iron compounds, depositing the iron as a waste product.

SUMMARY

Bacteria presenting the morphological characters of *Siderocapsa treubii* Molisch and *Siderocapsa major* Molisch were found upon glass slides submerged in various lakes and two streams. So many intermediate forms were encountered that it was considered impossible to separate these two species.

Siderocapsa treubii was present in all of the alkaline, hard water lakes of the drainage type, and absent in all of the neutral or acid, soft water lakes of the seepage type. It was found abundantly in two rapidly flowing streams.

Siderocapsa treubii is probably a heterotrophic organism utilizing the organic radicle of organic iron compounds and depositing the iron as a waste product upon the capsules of the colonies.

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PLATE 1

FIG. 1. A colony of *Siderocapsa treubii* with numerous coccoid cells.
FIG. 2. A colony with paired coccoid cells.
FIG. 3. A colony with rod-shaped cells.
FIGS. 4 and 5. Confluent or multiple colonies.

Fig.1   Fig.2   Fig.3

Fig.4   Fig. 5

(Yvette Hardman and Arthur T. Henrici: Studies of freshwater bacteria)
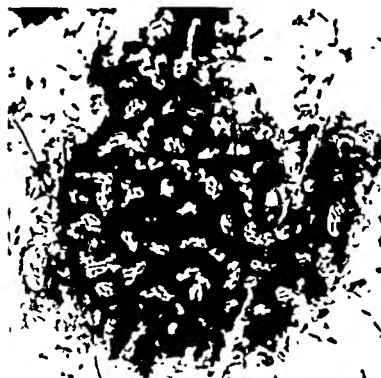
PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

First Meeting of the Current Season, Philadelphia County Medical Society Building, October 18, 1938, Philadelphia, Penna.

The Original Minutes of the Society of American Bacteriologists. *C. G. Roos*, Sharp and Dohme Laboratories, Glenolden, Penna.

Announcement was made of the finding of the original minutes of the first meetings of the Society of American Bacteriologists. While it was known that some records had been left at Glenolden in 1917 by the Society's secretary, Dr. A. Parker Hitchens, a post-war search had been fruitless. In the spring of 1938 the laboratories in the main building were remodeled and provision made for safe keeping of valuable records. While old records of value were being gathered, the record book containing the minutes of the Society's first meetings was brought to light.

The original minutes are contained in a small record book consisting of 200 pages. The 101 inscribed pages include the original transcript of the Society's constitution, minutes of the meeting of organization, names of the Society's organizers with a list of the names of its charter members, the first to the eleventh programs presented at the annual meetings and the minutes of transactions at those meetings. The earliest date recorded is October 16, 1899 and the last December 30, 1909.

Photostats are being made of the records and will be available at the library of the Mulford Biological Laboratories of Sharp and Dohme, Glenolden, Pa. The original record book will be delivered to the Archives Committee of the Society of American Bacteriologists.

The Pathogenesis of Local Tetanus. *William Chalian*, Laboratory for Endocrine Research, The Johns Hopkins University, School of Medicine, Baltimore, Md.

Theories of the pathogenesis of tetanus were reviewed. The nerve-blocking experiments of Meyer and the dynamic-activation experiments of Doerr and Seidenberg were shown to be defective and hence of no value to the axis-cylinder-carriage theory. The fact that denervated muscles fail to respond to the injection of tetanus toxin does not preclude the possibility of a peripheral action of this poison. The following theory is upheld: Tetanus toxin can be carried to the tissues of the body, whether these be specifically reacting striated muscles or motor horn cells or structures that do not respond in a recognizable manner to the action of the toxin, *only by the same mechanisms that effect the distribution of countless other drugs and poisons—the blood and the lymph-vascular systems*. The toxin is capable of exhibiting a dual action, the components of which are separately demon-

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strable. The first effect, which is a poisoning of the motor horn cells, results in the appearance of convulsions and reflex spasms. The second effect is exerted directly upon the motor end-organs present in striated muscle and is manifested by the development of muscular rigidity.

A detailed report of this work will appear shortly in the Bulletin of the Johns Hopkins Hospital.

RECENT ADVANCES IN OUR KNOWLEDGE OF TETANUS. *Warfield M. Firor, Johns Hopkins University, School of Medicine, Baltimore, Md.*

Long ago we became interested, with Dr. Abel, in the study of tetanus. Dr. Abel subjected the neural transport theory of Meyer to a critical review and concluded that tetanus toxin is absorbed by the lymphatics and distributed by the blood stream. He felt that the rigidity seen in local tetanus is due to a peripheral action of the toxin on the neuromuscular end-organs and that the hyperexcitable reflexes are central nervous system manifestations. To prove this latter point we injected into the gray matter of the lumbar cord of dogs minute amounts of tetanus toxin. With as little as 1/300th ordinary lethal dose we were able to produce tactile reflex tetanus. All these animals died. Further studies showed that following intraspinal injections, death results, regardless of whether the afferent and efferent nerves are severed or the spinal cord divided above the point of injection. These observations led us to hypothecate that tetanus toxin is altered to form a new substance. Experiments show that intravenous administration of tetanus antitoxin does not prevent death following the intraspinal injection of tetanus toxin. Experiments in which an injected animal is cross-circulated with a normal one seems to bear out the assumption that tetanus toxin is altered within the spinal cord to form a new substance.

ONE HUNDRED AND THIRTY-FOURTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PA., NOVEMBER 22, 1938

A SIMPLIFIED COMPLEMENT FIXATION TECHNIC FOR THE SEROLOGICAL DIAGNOSIS OF SYPHILIS. *Fred Boerner and Marguerite Lukens,* Graduate Hospital, University of Pennsylvania, Philadelphia, Pa.

A simplified technic for a qualitative or quantitative complement-fixation test is described which effects marked reduction in cost and labor. Chances of error are minimized by a reduction in the number of steps and by the fact that omission of any step is readily detected.

For the qualitative test, one dose of serum is employed; additional doses are used for the quantitative test.

Qualitative test: (1) Add 0.1 cc. of each serum to be tested to each of 2 tubes. Inactivate in water-bath, 58°C. for 10 min. Also include suitable controls. (2) To tube 1 add 0.5 cc. antigen-complement mixture (antigen diluted in 1:30 complement so 0.5 cc. contains proper dose of antigen). (3) To tube 2, add 0.5 cc. complement 1:30. (4) Mix tubes thoroughly, place in refrigerator (6°-10°C.), 15-18 hours. (5) Place in water-bath, 37°C., 10 minutes. (6) To both tubes add 0.5 cc. 0.75% sensitized corpuscles. (The corpuscles and hemolysin should be mixed at least 15 minutes before use.) (7) Mix thoroughly, place in water-bath, 37°C., for 1 hour. (8) Reactions are interpreted as follows: ++++,

positive; +++, ++, + or ±, doubtful; −, negative. (9) When several doses of serum are used for the purpose of making a quantitative determination of the degree of positiveness of the serum, the reactions obtained with each dose are recorded for comparison with previous or subsequent tests.

A SIMPLIFIED METHOD FOR THE PREPARATION OF AN ANTIGEN FOR USE IN COMPLEMENT FIXATION TEST FOR SYPHILIS. *Fred Boerner, Charles A. Jones and Marguerite Lukens*, Graduate Hospital, University of Pennsylvania, Philadelphia, Pa.

A simplified method for preparation of antigen for use in the above complement-fixation test for syphilis is described. (1) Place 10 gms. powdered beef heart (Difco) in 1 or 2 l. flask. (2) Add 225 cc. absolute alcohol and 75 cc. ether (U. S. P.); allow to stand at room T. $\frac{1}{2}$ hour, shaking thoroughly every 5 minutes. (3) Filter through filter paper, discard the residue. (4) Place the filtrate in large flask and evaporate, using slight suction, to 50 cc. by boiling in a water-bath. If the concentrated extract is less than 50 cc., add sufficient absolute alcohol to make up to volume. (5) Place the concentrated filtrate in the refrigerator for 1 hour. A heavy precipitate will form. (6) Filter through paper in the refrigerator so that the filtration will be completed while the solution is cold. (7) Allow the filtrate to stand at room T. overnight; if a precipitate forms, filter. (8) Dissolve 200 mgms. cholesterol in the 50 cc. of filtrate. This finished antigen is now ready to be tested for its optimum fixing dose and anti-complementary properties.

If desired that the antigen be more or less sensitive, merely vary the cholesterol content. Antigens prepared by the above method are slightly more anti-complementary than the alcohol used in their preparation, and their optimum antigenic dose is usually between 1:800 and 1:1,600. Any volume of antigen can be prepared by this method by proportionally increasing or decreasing the amounts.

REPORT OF THE CONFERENCE ON THE LABORATORY DIAGNOSIS OF SYPHILIS AT HOT SPRINGS, ARK., AND THE MEETING OF THE LABORATORY SECTION OF THE AMERICAN PUBLIC HEALTH ASSOCIATION IN KANSAS CITY, MO. *A. Parker Hitchens*, University of Pennsylvania, School of Medicine, Philadelphia, Pa.

WASHINGTON BRANCH

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., NOVEMBER 15, 1938

MECHANISM OF THE ACTION OF SULFANILAMIDE IN BRUCELLOSIS. *Henry Welch*, Food and Drug Administration, U. S. Department of Agriculture.

In cases of human brucellosis and in guinea pigs infected with *Brucella abortus*, treatment with sulfanilamide markedly increases phagocytosis of this organism. Individuals infected with other diseases and normal guinea pigs treated with sulfanilamide show no change in their opsonocytophagic activity for *Brucella abortus*. Further, the blood of normal human beings or normal guinea pigs, when treated *in vitro* with sulfanilamide, shows no change in opsonocytophagic activity

when mixed with untreated *Brucella abortus* or with sulfanilamide-treated organisms.

It has been possible in infected guinea pigs, treated with sulfanilamide, to demonstrate the absence of the drug in the blood at the time at which marked phagocytosis occurs, and, further, to transfer this marked phagocytic activity to normal guinea pig and human cells by suspending them in the serum of infected treated guinea pigs. Since the presence of sulfanilamide as such is not essential for the demonstration of marked phagocytosis in infected treated guinea pigs, it would appear that the effect of the drug is an indirect one.

The marked phagocytic activity demonstrated with normal cells in the presence of sera from infected individuals treated with sulfanilamide is completely lost by the addition to such sera of small amounts of a concentrated filtrate of *Brucella abortus*.

It would appear that sulfanilamide acts indirectly on *Brucella* infections by increasing the opsonic power of the blood and thus neutralizing the effect of the endotoxin or aggressin-like substances produced by this organism allowing phagocytosis to take place. The fact that it was not possible to stimulate the opsonic power of the blood toward *Brucella* of uninfected human beings or guinea pigs by treatment with sulfanilamide would indicate that an infective or immunizing process must be in progress for the drug to stimulate phagocytosis in this disease.

PROBLEMS IN PNEUMONIA. *Lloyd Felton*, National Institute of Health.

PRECURSORS TO THE FORMATION OF CREATININE BY BACTERIA

C. H. FISH AND T. D. BECKWITH

Department of Bacteriology, University of California at Los Angeles

Creatinine is a urinary excretory product from warm-blooded animals but the mode of its formation is not, as yet, completely understood. Folin (1905) determined that its production was independent of intake of food and protein and of non-protein nitrogen. Moreover, its daily output is normally constant. The amount of creatinine excreted per day is proportional to the muscular development of the individual as shown by Tracy and Clark (1914).

Since creatinine may be considered to be dehydrated creatine, it has been assumed that creatine is its forerunner, although findings here have been diverse (Folin, 1914 and Lyman and Bundy, 1917). In addition, arginine, choline, glycine-betaine and cystine have been advanced as precursors of creatine but final evidence of the effectiveness of anyone of these is lacking. It is possible that they may be only stimulants. The complexities of the animal body are such that evaluation of variables frequently becomes impossible and therefore it was decided to turn to simple organisms for our experiments, which have been done with certain bacteria.

Fitzgerald and Schmidt (1912) and Sears (1916, 1917) have demonstrated that bacteria can produce creatinine and the latter showed that *Proteus vulgaris* formed this compound from peptone. In the presence of glucose, with the peptone, the amount of creatinine appearing was increased. Because of the relatively simple structure of the bacterial media, control of variables is easier than in the body of an animal.

Creatinine may be related theoretically to acetic acid, glycine,

sarcosine, guanidine, methylguanidine, glycocyamine, arginine, urea or choline and certain of these were examined to determine their availability to act as precursors of creatinine when exposed to the growth activities of bacteria.

Procedure. Following our introductory experiments which were devoted to verification of the observations of Sears (1916, 1917), synthetic media were utilized. The common base for these media was Mueller's (1935) salt solution containing $NaCl$, K_2HPO_4, $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$ dissolved in distilled water. This solution was sterilized in the Arnold Steamer. The following amino acids have been included: glycine, dl-alanine, dl-glutamic acid, d-arginine HCl, cystine, dl-phenylalanine, dl-tyrosine, dl-aspartic acid, dl-leucine, and these compounds were obtained from Amino Acid Manufactures at the University of California at Los Angeles and were stated to contain not more than 0.004 per cent of impurities. These substances were used in watery solutions and the concentration of hydrogen-ion was adjusted to 7.0. Sterilization was effected by the intermittent method. Media under test were compounded at time of use by addition of 0.25 cc. of Mueller's solution to 10 cc. of solution of the amino acid. Culture volumes usually were 60 cc.

The organisms were from stock cultures in the department of the University and had been previously isolated at various times.

Determinations of creatinine were made by the method of Jaffé (1886). A solution of creatinine chloride of concentration of 100 mlg. creatinine in 100 cc. of distilled water was standardized against one of potassium dichromate. In turn, this was diluted to suit subsequent tests. Saturated aqueous solution of picric acid was tested for purity by the method of Folin and Doisy (1917). A representative test was conducted in the following manner: Five cc. of culture solution plus 5 cc. of alkaline picrate stood for five minutes and then were diluted volumetrically to 25 cc. with distilled water. By a colorimeter this solution was compared with 5 cc. of the standard dilution of creatinine to which had been added 5 cc. of alkaline picrate. This, after standing for five minutes, in turn was diluted volumet-

rically to 25 cc. with distilled water. If culture solutions were turbid, they were centrifugalized to prevent interference with readings in the colorimeter.

EXPERIMENTAL

Experiment 1. Since Sears (1916) stated that *Staphylococcus aureus, Proteus vulgaris* and *Escherichia coli* produce creatinine in peptone water and that this creatinine is increased in amount in the presence of glucose, his work was duplicated with results

TABLE 1

Production of creatinine by three organisms listed

Amounts are expressed as milligrams in 100 cubic centimeters of media

	E. COLI		S. AUREUS		P. VULGARIS	
	3 days	14 days	3 days	14 days	3 days	14 days
Peptone water........................	4.8	5.2	3.5	3.3	5.1	5.0
Peptone water + 1 per cent glucose....	5.6	6.0	4.0	3.4	6.5	6.8

TABLE 2

Production of creatinine by three organisms listed

Amounts are expressed as milligrams in 100 cubic centimeters of media.
Incubation period was four days

	0.1 PER CENT GLYCINE	0.5 PER CENT GLYCINE
S. aureus	No growth	No growth
E. coli	0.40	0.40
P. vulgaris	0.45	0.50
Solution of sodium picrate	0.37	

indicated in table 1. Thus, the work of Sears (1916) has been confirmed and it is indicated that *P. vulgaris* is more active than *E. coli* or *S. aureus* in formation of creatinine.

Experiment 2. Since peptone contains a complex of amino acids, the next step was to consider one of the simplest of these, glycine, as a source for formation of creatinine when the three organisms used in experiment 1 were placed in contact with it. For this purpose solutions of glycine of 0.1 and 0.5 per cent concentration were utilized. Results are set forth in table 2.

Since sodium picrate, because of the color of its solution, gave an apparent reading of 0.37, it is obvious that a trace only of creatinine was produced in this test. These results indicated that further work was likely to deal with small differences and caused us in certain instances to modify the technique by adding standard creatinine solution to the unknown. The procedure of McCrudden, and Sargent (1916) as indicated below, was employed;

Unknown	*Standard*
5 cc. culture solution	5 cc. distilled water
5 cc. standard solution	5 cc. standard solution
5 cc. alkaline picrate	5 cc. alkaline picrate
Wait 5 minutes	
Dilute to 25 cc. volumetrically	
Read by colorimeter	

In this experiment, glycine served as the sole source of C and N for the bacteria and of itself was insufficient for formation of creatinine.

Experiment 3. Glycine corresponds only to that portion of the molecule of creatinine which contains $\diagdown\text{N—CH}_2\cdot\text{COOH}$. Since glycine can serve in such limited degree, it is likely that a precursor corresponding to the $\text{HN}{=}\text{C—NH}_2$ portion of the molecule of creatinine might be lacking. Urea might serve this purpose. Therefore various combinations of solutions of glycine, urea, ammonium sulphate and glucose were combined to constitute the series presented in table 3 with *Proteus vulgaris* to serve as the test organism. Ammonium sulphate was introduced into this series to serve as a control. To include ammonium carbonate would have incorporated an additional source of carbon and that was undesirable.

A portion of each of these formulae was kept uninoculated until the conclusion of the experiment and then was tested for possible presence of creatinine. Results were negative in each instance.

Since the results obtained by use of glycine, urea and glucose were so exceptional when compared to other units of the series,

the work with this particular combination was repeated. Readings were made at frequent intervals during incubation at 37°C. Creatinine appeared in a concentration of 1.6 milligrams per 100 cubic centimeters in twenty-four hours. At five days, it was 6.66 and at fourteen days, 5.0.

A mixture of glycine, urea and glucose produced the largest yield of creatinine by *Proteus*; while all other solutions used permitted formation of little or none of this by-product.

Experiment 4. Arginine contains the guanidine group and thus likewise may serve as a precursor to the formation of cre-

TABLE 3

Production of creatinine by Proteus vulgaris when in presence of glycine, urea ammonium sulphate and glucose in various combinations

Results indicated as milligrams per 100 cubic centimeters

MEDIUM	4 DAYS	7 DAYS	14 DAYS
0.1 per cent glycine...................................	0	0.1	0.1
0.1 per cent glycine + 1 per cent glucose............	0.16	0.44	0.32
0.1 per cent glycine + 0.5 per cent urea.............	0.1	0.1	0.1
0.1 per cent glycine + 1 per cent glucose + 0.5 per cent urea	Not tested	13.3	3.0
0.1 per cent glycine + 1 per cent glucose + 0.5 per cent ammonium sulphate............................	Not tested	0.22	0
0.1 per cent glycine + 0.5 per cent ammonium sulphate.	0.16	0.1	0.1
1 per cent glucose + 0.5 per cent urea.................	0	0	0
0.5 per cent urea (no growth)........................	0	0	0
0.5 per cent ammonium sulphate + 1 per cent glucose ..	0	0	0
0.5 per cent ammonium sulphate (no growth).........	0	0	0

atinine. Therefore the series presented in table 3 was set up, with arginine substituted for glycine. With the exception noted, all conditions were identical in the two experiments. The results of these tests are presented in table 4. It may be concluded that arginine is not so effective as is glycine for production of creatinine by *Proteus*. Moreover an examination of tables 3 and 4 indicates that in the presence of the amino acid and glucose, ammonium sulphate cannot replace urea for formation of creatinine.

Experiment 5. Inasmuch as glycine and arginine may serve

with urea and glucose as sources for formation of creatinine, it was determined to examine other amino acids individually for like effect. These were incorporated with 0.5 per cent urea and 1 per cent glucose in the synthetic medium described previously and the results are shown in table 5.

TABLE 4

Production of creatinine from d-arginine HCl by Proteus

Concentrations presented as milligrams per 100 cc. medium

MEDIUM	3 DAYS	7 DAYS	14 DAYS
0.1 per cent d arginine HCl.................................	0.1	0	0.15
0.1 per cent d arginine HCl + 1 per cent glucose..........	0.24	0.52	0.52
0.1 per cent d arginine HCl + 0.5 per cent urea...........	0	0	0
0.1 per cent d arginine HCl + 0.5 per cent ammonium sulphate...	0	0	0
0.1 per cent d arginine HCl + 0.5 per cent urea + 1 per cent glucose...	0.28	0.30	1.2
0.1 per cent d arginine HCl + 1 per cent glucose + 0.5 per cent ammonium sulphate...............................	0	0	0

TABLE 5

Production of creatinine by Proteus from a series of amino acids in presence of glucose

Results are expressed as milligrams per 100 cc. of medium

AMINO ACID IN MEDIUM	3 DAYS	14 DAYS
0.1 per cent Glycine..........................	4.17	4.0
0.1 per cent dl Alanine.......................	3.46	2.0
0.1 per cent dl Glutamic acid.................	2.50	1.5
0.1 per cent Cystine..........................	3.90	4.6
0.1 per cent Phenyl alanine...................	2.80	2.08
0.1 per cent dl Leucine.......................	2.5	2.3
0.1 per cent dl Tyrosine......................	2.8	3.46
0.1 per cent dl Aspartic acid.................	2.08	3.9
0.1 per cent d Arginine HCl...................	2.30	2.0

Evidently creatinine can be formed by *Proteus* from all of these amino acids although glycine, cystine, aspartic acid and tyrosine are the most effective of the series.

Experiment 6. In the work described thus far, glucose has been the form of carbohydrate utilized. It was desirable to

determine whether another carbohydrate may be substituted for this hexose. A series identical to that given in table 3 except that a pentose, arabinose, replaced glucose was therefore inoculated with *Proteus*. All other conditions were similar. Final readings with the combination of glycine, urea and arabinose indicated the appearance of 0.22 milligram of creatinine per 100 cubic centimeters rather than 3.0 which were obtained when glucose was present. Subsequent work showed galactose to afford 40 to 65 per cent of the effectiveness of glucose. Maltose upon hydrolysis yields two molecules of glucose but when this

TABLE 6

Production of creatinine by bacteria in media described

Readings in terms of milligrams per 100 cc. of solution

ORGANISM	PEPTONE WATER	PEPTONE WATER + 1 PER CENT GLUCOSE	0.1 PER CENT GLYCINE + 1 PER CENT GLUCOSE + 0.5 PER CENT UREA	0.1 PER CENT D ARGININE HCl + 1 PER CENT GLUCOSE + 0.5 PER CENT UREA
Proteus	4.0	5.4	4.1	2.7
Proteus × 19	5.0	3.5	4.4	2.03
Alcaligenes	4.6	4.4	2.7	2.0
Mycobacterium phlei	3.3	4.1	1.2	2.05
Mycobacterium smegmatis	3.7	4.0	1.4	2.00
Bacillus subtilis	3.9	3.7	2.0	1.98
Aerobacter aerogenes	3.9	4.1	2.2	2.23
Escherichia communis	4.8	5.0	1.7	2.68
Escherichia communior	4.8	4.8	1.8	1.46
Escherichia lactici-acidi	4.0	4.1	1.6	2.05

disaccharide in turn replaced glucose in the media, the yield of creatinine was but 67 per cent of that produced by the monosaccharide. In further work arginine was substituted for glycine and arabinose for glucose. Again it was indicated that the presence of glucose is essential for production of the larger amounts of creatinine.

Experiment 7. Three organisms were considered in the first studies, while *Proteus* alone was used for those designed to determine production of creatinine. It was decided to amplify these findings by inclusion of additional forms. For this purpose, ten bacterial species were selected and with these was incor-

porated *Proteus vulgaris* to serve as a standard for comparison. Four media were utilized. Final readings of the concentration of creatinine and description of the media are presented in table 6. Incubation extended over a period of fourteen days at 37°C. These results indicate that creatinine in considerable concentration may be produced by a variety of bacterial species. With one exception it is demonstrated that more of the by-product is formed in the presence of peptone than with glycine alone and that in many instances, formation of creatinine is intensified by the presence of glucose.

Experiment 8. It was demonstrated in table 4 that small amounts of creatinine may be produced from arginine. The presence of urea was repeatedly shown to be necessary if con-

TABLE 7

The effect of manganese sulphate upon production of creatinine by Proteus
Concentrations noted as milligrams per 100 cc. of medium

MEDIUM	3 DAYS	14 DAYS
0.1 per cent d Arginine HCl + 1 per cent Glucose..........	0.24	0.52
0.1 per cent d Arginine HCl + 1 per cent glucose + 5 per cent of 0.1 Mol. MnSO₄.....................................	3.56	3.12
0.1 per cent Glycine + 1 per cent Glucose + 0.5 per cent urea..	2.3	5.0
0.1 per cent Glycine + 1 per cent Glucose + 0.5 per cent urea + 5 per cent 0.1 Mol. MnSO₄...........................	4.17	5.0

siderable concentrations of this compound are to appear. Arginine contains within its molecular structure two portions: a guanidine group $HN = C \begin{matrix} HN_2 \\ NH \end{matrix}$ and a moiety of urea $=$

$C \begin{matrix} NH_2 \\ NH \end{matrix}$. The question then arises as to whether either of these

is related to the production of creatinine by *Proteus*. Arginase breaks down arginine with the formation of urea and ornithine. Thus, it destroys the guanidine fraction. Hellerman and Perkins (1935) have demonstrated that salts of manganese and of cobalt

may serve as activators of arginase. It was determined to observe whether the presence of an activator of arginase would stimulate formation of increased amounts of creatinine.

Preliminary trials indicated that the cobalt would interfere but that manganese would not interfere with the reading of the reaction. Therefore, the following series presented in table 7 was set up and results as indicated after fourteen days incubation at 37°C. were obtained.

From these data it is evident that the destruction of the guanidine portion of the molecule of arginine with resultant formation of urea gives rise to the appearance of increased concentration of creatinine when exposed to the enzymatic action of *Proteus*. Additional evidence regarding the participation of urea in formation of creatinine is adduced.

DISCUSSION AND CONCLUSION

Creatinine can be produced from peptone by a variety of bacteria but the peptone must be considered as a complex structure involving many factors. Further consideration has proven that creatinine may be formed through bacterial action from various amino acids when glucose is present. In seeking for its precursors, creatinine has been treated as a compound containing two critical portions which are (1) acetic acid and (2) guanidine. It has been shown that glycine, urea and glucose when under the influence of *Proteus vulgaris* give rise to considerable concentrations of creatinine. Other amino acids than glycine supply either the acetic acid moiety or urea necessary for the reaction. Added evidence regarding the importance of urea is presented by treatment of arginine by an activator of arginase. For production of creatinine by *P. vulgaris* urea may not be substituted by a salt of ammonium. The hexose glucose is the form of carbohydrate most readily utilized by the organism in the course of creatinine production. The precursors of creatinine as produced by *Proteus* are acetic acid moiety and urea or glycine and urea.

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A COMPARATIVE STUDY OF THE USE OF VARYING CONCENTRATIONS OF AGAR IN THE TEST MEDIUM USED TO DETECT CONTAMINANTS IN BIOLOGIC PRODUCTS[1]

CAROLYN R. FALK, HELEN BIENIASZ BUCCA, AND
MARGARET P. SIMMONS

Bureau of Laboratories of the Department of Health of the City of New York

INTRODUCTION

Previous reports on the bactericidal action of antiseptics used in biologic products (Rosenstein and Levin, 1935; Falk and Aplington, 1936) showed that, when a given organism was inoculated into both broth and agar media, growth sometimes appeared in the agar and not in the broth and vice-versa. These findings could not be attributed to bacteriostatic action caused by insufficient dilution of the antiseptic present, since identical results were at times obtained in the control tubes containing no antiseptic. It was therefore indicated that neither broth nor agar used alone could be relied upon to detect organisms which may be found as contaminants (staphylococci, diphtheroids, and *Pseudomonas pyocyaneus*) in biologic products. These observations were later corroborated by Eldering and Kendrick (1936).

A semifluid medium, 0.1 per cent agar, was recommended by Hitchens (1921) for the detection of bacterial contaminants, either aerobic or anaerobic, particularly in substances containing a high percentage of antiseptic. While this medium has not attained the universal use that 0.03 per cent glucose broth has for testing the sterility of biologic products, it has been used in conjunction with this broth by Wadsworth (1927) and others.

More recently, Spray (1936) reported that anaerobes previously considered difficult to cultivate, grew well on a semisolid medium without a seal. We have not found, however, any extended experimental or statistical studies which demonstrated that a semifluid, semisolid, or solid medium would be preferable to broth for the detection of the more common forms of bacterial growth, or as a substitute for the regular 0.03 per cent glucose broth for testing the sterility of biologic products. It seemed, therefore, advisable to make such a study, particularly since the use of two very different types of medium is obviously undesirable in any laboratory concerned with large scale production of biologic products.

EXPERIMENTAL METHOD

The efficiency of different concentrations of agar in the medium used for the detection of bacterial growth was evaluated by (1) adding organisms directly to the test media; (2) adding organisms to typical biologic products, containing preservative, and subculturing to the various test media; and (3) adding a biologic product known to have a bacterial contaminant to the various test media.

Concentrations of agar in the test media varied from 2.0 to 0.001 parts of agar per hundred (or one to 2000 parts of agar per 100,000). The media containing from one to 0.001 parts of agar per hundred were prepared by diluting 2 per cent biologic-products agar with sufficient biologic-products broth (containing 0.03 per cent glucose) so that the desired concentrations of agar were obtained. The method of preparing these basic media has been described in previous papers (Rosenstein and Levin, 1935; Falk and Appington, 1936). The media, made up in 300 cc. batches, were tubed aseptically in 6″ x $\frac{5}{8}$″ tubes, 10 cc. being delivered into each tube. Immediately before use the test medium was heated at 100°C. in the Arnold sterilizer for one hour, followed by quick cooling to 45°C. As in our previous studies, the test organisms were types which may be encountered as contaminants in biologic products. In fact the strains used were actually isolated from such products over a period of years.

In the experiments to be described here, the staphylococci, diphtheroids, and *P. pyocyaneus* previously used were supplemented by hay bacilli, streptococci and *Escherichia coli*. Eighteen-hour broth cultures, containing approximately 100,000,000 organisms per cubic centimeter (colony counts varied from 60-000,000 to 250,000,000), of the above-mentioned organisms were diluted with broth so that the 10^{-4} dilution contained approximately 10,000 organisms per cubic centimeter, the 10^{-5} dilution 1000 organisms per cubic centimeter, the 10^{-6} dilution 100 organisms per cubic centimeter and the 10^{-7} dilution 10 organisms per cubic centimeter. A plate count was made to determine the exact number of organisms present in every experiment. One-tenth of a cubic centimeter of the appropriate dilution was then added to each of the various test media directly or to typical biologic products, which were subcultured to the test media. These tests were then incubated at 36°C. for seven days, during which time observations were made daily. All tests were carried out in duplicate, each complete series of tests being repeated at least once. The identity of organisms in tubes showing growth was checked by smears stained by Gram's method.

EXPERIMENTAL RESULTS

Typical results are given in tables 1 to 3. In the first series of experiments, in which organisms diluted with broth were used, there was a striking variation in the rate and character of the growth of the organisms in the various test media. These differences were apparently dependent on the percentage of agar present. Growth was inhibited when the percentage of agar was too high as well as when the medium was too fluid (table 1). In all cases, when the concentration of agar in the medium was between 0.06 and 0.50 parts per hundred, growth was most luxuriant. This fact became increasingly evident as the size of the inoculum decreased. In fact, when the medium contained concentrations of agar between 1.0 and 0.12 per cent, the presence of very few organisms could be demonstrated, whereas negative results were obtained in the media containing either

TABLE 1

The effect of different concentrations of agar on the growth of organisms when varying inoculums are used

7 day readings

AGAR per cent	HAY BACILLI DILUTED			STAPHYLOCOCCI DILUTED			P. PYOCYANEUS DILUTED			DIPHTHEROIDS DILUTED		
	10^{-3}	10^{-5}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}
2.0	++	++	–	++	++	++	++	++	–	–	–	–
1.0	++	++	++	++	++	++	+++	+++	+	+	+	+
0.50	++++	+++	++	++++	++++	++++	++++	++++	+	++++	++++	+
0.25	++++	++++	++++	++++	++++	++++	++++	++++	+	++++	++++	++
0.12	++++	++++	++++	++++	++++	++++	++	++	–	++	++	+
0.06	+++	+++	+++	++	++	–	++	++	–	++	++	+
0.03	+++	+++	++	+++	+++	–	+	+	–	+	+	+
0.015	++	++	+++	+++	+++	–	+++	+++	–	+++	+++	+
0.008	++	++	+++	+++	+++	–	+++	+++	–	+++	+++	+
0.001	++	+++	+++	++	++	–	+++	+++	–	+++	+++	+
0.002	++	+++	+++	+	++	–	+++	+++	–	+++	+++	+
0.001	+	+++	+	–	+	–	+++	+++	–	+++	+++	+
0.000	+				–	–	+++	+++	–	+++	+++	+

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; –, no growth.

more or less agar in many cases. As might be expected, individual preferences for definite concentrations of agar in the medium were shown by the different test organisms. For example, staphylococci inoculated in small numbers did not grow in a medium containing less than 0.25 per cent agar, diphtheroids if the medium contained more than 1 per cent agar, P. *pyocyaneus* if more than 1 per cent or less than 0.25 per cent agar was present; hay bacilli on the other hand, showed growth in all the test media, but this growth was most luxuriant when the medium contained between 0.06 and 0.25 per cent agar.

To determine the practical application of these results, another series of experiments was carried out in which actual routine working conditions were more nearly approximated. Results obtained when test organisms were added to typical biologic products are shown in table 2. Typical products included a bacterial vaccine, an antitoxic globulin, and an antibacterial serum. Eighteen-hour broth cultures of each of the organisms were diluted with broth so that each cubic centimeter contained 10,000 organisms; 0.5 cc. of this dilution was then added to sterile 5 cc. samples of the above named products. Subcultures were made to the various test media after the culture had been in contact with the product for thirty minutes, since in most cases the preservative present might be expected to destroy the test organisms on continued standing. Again as in our first experiments, maximum growth was obtained when the concentration of agar in the medium varied from 0.06 to 0.50 per cent. The exact optimum concentration of agar needed, as before, depended on the type organisms present. The intermediate environment to which the organisms had been subjected, also, seemed to influence the concentration of agar in the medium which produced the most luxuriant growth on subculture. Organisms added to the bacterial vaccine corresponded most closely to the broth control, most luxuriant growth occurring in media containing from 0.12 to 0.5 per cent agar. On the other hand, organisms added to the antitoxic-globulin needed a more fluid medium, that is, one containing from 0.12 to 0.06 per cent of agar; and organisms added to the bacterial serum

TABLE 2

The effect of different concentrations of agar on the growth of organisms

Readings made after incubation at 36°C. for 96 hours

AGAR	HAY BACILLI				STAPHYLOCOCCI				P. PYOCYANEUS			
	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin
per cent												
2.0	++	++		+	++	++	−	+	++	++	++	+
1.0	+++	++	++	++	++	++	+	+++	+++	+++	++	+
0.5	++++	+++	+++	+++	++	+++	++	+++	+++	++++	+++	+++
0.25	++++	++++	+++	+++	+++	+++	+++	++++	++++	++++	+++	++++
0.12	++++	++++	++	++++	++++	++++	++++	++++	++++	++++	+++	++++
0.06	+++	++	−	++	++	++	−	++	++	+++	++	++
0.03	++	++	−	++	++	+	−	++	++	++	++	++
0.000		+	−				−					

AGAR	DIPHTHEROIDS				STREPTOCOCCI				E. COLI			
	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin
per cent												
2.0	++	++	++	++	++	++	++	++	++	++	++	++
1.0	++	++	++	++	++	++	++	++	+++	+++	++	+++
0.5	+++	+++	+++	+++	+++	++	+++	++	+++	+++	+++	+++
0.25	++++	++++	+++	++++	+++	+++	+++	+++	++++	++++	++++	++++
0.12	+++	+++	−	+++	+++	+++	++	+++	++++	++++	+++	++++
0.06	++	++	−	+++	++	++	−	++	+++	+++	+	+++
0.03	++	+	−		+	+	−		++	+	−	++
0.00	++		−						++		−	++

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; −, no growth.

TABLE 3

The comparative efficiency of different concentrations of agar in the sterility test medium when gram-positive aerobic bacilli were present in a known contaminated product

AGAR per cent	Vial 1			Vial 2			Vial 3			Vial 4			Vial 5		
	2	4	7	2	4	7	2	4	7	2	4	7	2	4	7
2.0	−	+++ / +++	++++ / +++	−	+++ / +++	+++ / +++	−	+++ / +++	+++ / +++	−	+++ / +++	++ / +++	−	++ / +++	++ / +++
1.0	+	+++ / +++	+++ / +++	+	+++ / +++	+++ / +++	+	+++ / +++	+++ / +++	+	+++ / +++	+++ / +++	+	+++ / ++++	+++ / ++++
0.5	++	+++ / ++++	+++ / ++++	++	+++ / ++++	+++ / ++++	++	+++ / +++	+++ / +++	++	+++ / +++	+++ / +++	−	+++ / ++++	+++ / ++++
0.25	+++	+++ / ++++	+++ / ++++	+++	+++ / ++++	+++ / ++++	+++	+++ / ++++	+++ / ++++	++	+++ / ++++	+++ / ++++	+++	+++ / ++++	+++ / ++++
0.12	+++	+++ / ++++	+++ / ++++	+++	+++ / ++++	+++ / ++++	+++	+++ / +++	+++ / +++	++	+++ / +++	+++ / +++	+++	+++ / +++	+++ / +++
0.06	+++	+++	+++ / +++	+++	+++	+++ / +++	+++	++	+++ / +++	++	+++	+++ / +++	++	+++	+++ / +++
0.03	+++	++	+++ / +++	+++	+++	+++ / +++	++	+++	+++ / +++	+	+++	+++ / +++	++	+++	+++ / +++
0.00	−	−	+*	−	−	−	−	−	−	−	−	−	−	−	−

DAYS INCUBATED AT 36°C.

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; −, no growth.

* Growth observed in only one of two similarly inoculated tubes.

seemed to favor the media with concentrations of agar varying from 0.12 to 0.5 per cent, often giving negative results if less than 0.1 per cent agar was present. However, irrespective of the type of organism or its environment, optimum growth was regularly obtained in the media containing from 0.12 to 0.25 per cent of agar.

Soon after the completion of these experiments, we were able to secure several vials of a product which had become contaminated with a gram-positive aerobic bacillus in the process of manufacture. This particular product had shown growth in only one broth fermentation tube out of the twenty-five inoculated in routine sterility tests. The poured agar plates also showed no growth. For the experimental series of tests, 0.1 cc. portions from each vial were added to each of two tubes containing 10 cc. of test medium. Results of these tests are given in table 3. Again, only one of the ten broth tubes inoculated showed growth, while good growth was observed in every tube in which the medium contained between 1.0 and 0.1 per cent of agar. This experiment also demonstrated that the time at which growth was first observed in any one tube was influenced by the concentration of agar present. Good growth was first noticed in 48 hours in media containing concentrations of agar varying from 0.12 to 0.25 per cent, but it required at least 96 hours to show the same type of growth in media containing more than 0.25 per cent agar or less than 0.12 per cent agar, and as much as seven days when broth alone was used.

PRACTICAL APPLICATION

The experiments which have been described all showed that bacterial growth could be readily detected in media containing between 0.1 and 0.2 per cent agar. It therefore seemed advisable to use this medium in conjunction with the regular biologic-products broth for the routine testing of sterility of biologic products. All sterility tests were carried out following the recommendations of the National Institute of Health, with the exception that one half of the volume of product to be tested was added to the broth in fermentation tubes, while the re-

mainder was added to the 0.1 per cent agar medium.   For
routine purposes, the medium was dispensed in 7″ x 1″ metal
capped tubes, each tube containing 35 cc. of medium.   This
medium received the same preparatory treatment as the
biologic-products broth in fermentation tubes, that is, heating
at 100°C. for one hour in the Arnold sterilizer, within two
hours before use, followed by rapid cooling to 45°C.   As has
been pointed out by Wadsworth (1927), it is of the utmost
importance that the 0.1 per cent agar medium be clear, so that
individual colonies may be observed throughout the medium as

TABLE 4

*The comparative efficiency of biologic-products broth and 0.1 per cent agar for the
detection of contaminants in biologic products*

LOT	B.P. BROTH			0.1 PER CENT AGAR		
	Tubes inoculated	Tubes contaminated	Contamination	Tubes inoculated	Tubes contaminated	Contamination
			per cent			*per cent*
a	42	42	100	70	53	76
b	30	3	10	30	24	80
c	60	17	28	100	76	76
d	60	38	63	100	14	14
e	72	8	11	120	71	59
f	92	1	0.9	130	34	26
g	36	0	0.0	60	9	15
h	100	2	2.0	120	19	16
i	36	1	2.8	60	5	8.3
Total......	528	112	21.2	790	305	38.6

well as growth on the surface.   Cloudy media necessitate sub-
culture or the preparation of smears, in which case, any dead
organisms present may cause confusion.   To facilitate the in-
oculation and reading of tests, whenever possible, the broth and
0.1 per cent agar tubes were arranged in parallel rows in monel
metal racks constructed for this purpose.   Careful statistics
were kept on the results obtained with these two media in the
routine testing of the sterility of biologic products for a period
of eight months.   The comparative efficiency of the biologic-
products broth and 0.1 per cent agar in the tests which showed
bacterial contamination are given in table 4.   Each lot showing

bacterial growth was retested at least once. The contaminating organisms in these lots were Gram-positive aerobic bacilli. The adoption of the mixture of phenol and merthiolate, as a preservative in most biologic products has markedly reduced the contaminations caused by *P. pyocyaneus*, diphtheroids and staphylococci, but this mixture is not effective against sporebearing organisms of the hay bacillus group. In general, growth was demonstrated in approximately twice as many of the 0.1 per cent agar tubes as in the biologic-products broth tubes. If only a light contamination was present, few if any broth tubes showed growth, in contrast to from 8 to 26 per cent of the tubes containing 0.1 per cent agar. In fact, in one of these nine lots showing growth, the product would have been released as sterile had only broth fermentation tubes been inoculated. On the other hand, where the contamination was extensive, growth was observed in both types of medium, but in most cases growth appeared earlier and was detected in more of the 0.1 per cent agar tubes. This was, however, reversed in two cases, one in which slightly more of the broth tubes showed growth, and another in which the apparent difference was more significant, only 14 of the 0.1 per cent agar tubes showing growth in contrast to 63 per cent of the broth tubes. We have also observed that the inclusion of the 0.1 per cent agar medium offers a great advantage over the exclusive use of biologic-products broth in fermentation tubes in that this medium contains just enough agar to permit the formation of separate colonies, thus permitting a quantitative estimate of the extent of the contamination present.

SUMMARY AND CONCLUSIONS

These experiments demonstrate the value of using a medium containing small percentages of agar (0.06 to 0.25 per cent) for the detection of bacterial growth. This applies even to such common forms as hay bacilli and staphylococci, which are ordinarily considered easy to cultivate. Since little difference in growth could be detected in media containing between 0.1 and 0.25 per cent agar, 0.1 per cent agar was chosen for com-

parative tests with 0.03 per cent glucose biologic-products broth for the routine testing of biologic products for sterility. (It is of interest as has been mentioned in the introduction to note that this medium was suggested by Hitchens in 1921, but did not obtain the universal acceptance that biologic-products broth did.) The results of our routine tests with 0.1 per cent agar have confirmed our experimental findings, as well as confirming and extending those of Hitchens (1921) and Spray (1936). The adoption of a semifluid medium as a standard means of detecting and giving a quantitative estimate of the extent of bacterial growth in biologic products can therefore be recommended. Applications to other materials and procedures involving bacterial growth are also indicated.

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DOUBLE-ZONE BETA-HEMOLYTIC STREPTOCOCCI

Their Cultural Characteristics, Serological Grouping, Occurrence and Pathogenic Significance

J. HOWARD BROWN

Department of Pathology and Bacteriology, Johns Hopkins University, Baltimore, Md.

The appearance of double-zone beta-hemolytic streptococci in blood agar has been described in a previous publication (Brown, 1937b). If this striking appearance were one which might be displayed by various hemolytic streptococci it would be nothing more than an interesting phenomenon. Since it is found to be correlated with certain cultural reactions and serological groupings it assumes systematic and diagnostic significance. Its practical value is enhanced because it often enables these streptococci to be recognized in the primary blood-agar plate, even before pure cultures have been obtained.

This report embraces a study of 188 strains, of which 138 are from human sources and 50 of animal origin. Eighty-six strains were received from other workers (R. C. Lancefield, H. Plummer, R. B. Little, G. J. Hucker, L. Kirschner, J. M. Murphy, W. D. Frost, J. M. Rosell, E. I. Parsons). Of these strains 55 were from Lancefield, including 33 isolated by Ronald Hare, 6 by M. H. Dawson and 10 by W. N. Plastridge, the remaining 102 strains were isolated in the author's laboratory.

As indicated in a previous publication (Brown, 1937a), three fermentative groups of double-zone beta-hemolytic streptococci were found with reference to lactose and salicin: lactose −, salicin +; lactose +, salicin +; and lactose +, salicin −. All strains hydrolysed sodium hippurate and fermented glucose, sucrose and trehalose; none fermented mannitol, raffinose, inulin

or sorbitol. The final pH in glucose broth was 4.3 to 4.9; all but three of the strains within 4.4 to 4.6. Eight strains, including representatives of the three fermentative groups, were tested for the fermentation of other substances and all gave the following results: levulose, galactose, maltose, glycogen and dextrin fermented; inositol, adonitol, dulcitol, xylose, arabinose, rhamnose and esculin not fermented.

For the determination of the fermentation reactions of streptococci, three precautions are regarded as essential: the basic medium must be one which supports good growth of the organisms in the absence of fermentation; the test sugars, other than glucose, must not be sterilized in the medium; the cultures must be incubated sufficiently long to detect slow fermentations. In these studies, the basic medium has been broth made from fermented meat infusion to each tube of which 3 or 4 drops of sterile ascitic fluid were added aseptically. The carbohydrates (5 or 10 per cent solutions in distilled water) were sterilized in the autoclave or by filtration and added aseptically to the sterile fermented broth. Before inoculation the media were incubated to determine sterility. The cultures were incubated for at least five days before final readings were made by determining the pH of the glucose broth cultures and noting the color of the brom-cresol-purple which was used as an indicator in the other sugar media. The importance of the above precautions was demonstrated by the study of a number of strains received from other laboratories and reported in the literature as fermenting lactose when tested in Hiss serum water. When these strains were retested by us, they fermented lactose which had been added to Hiss serum water before sterilization, but in Hiss serum water and in fermented infusion broth with lactose added aseptically, there was no fermentation, although good growth occurred.

The hydrolysis of sodium hippurate was determined in infusion broth containing 1 per cent of sodium hippurate. Before testing with ferric chloride reagent, distilled water was added to the previously marked tubes to replace water lost by evaporation during storage and incubation. By observing this precaution, and by using a properly balanced and tested reagent, we have never failed to obtain definite and consistent results.

Twenty-seven strains of double-zone hemolytic streptococci were inoculated onto 40-per cent bile agar. In comparison with

hemolytic streptococci of serological groups A and C most of the strains grew well although a few showed only meagre growth. Our impression is that medium containing 40-per cent bile is not sufficiently differential for final differentiation of the streptococci.

Twenty-one of the double-zone strains of human and bovine origin and representing all of the three fermentative groups were tested for fibrinolytic activity against human plasma by the technique of Tillett and Garner (1935). None was fibrinolytic.

White mice were inoculated intraperitoneally with 0.5 cc. of over-night ascitic-fluid broth cultures of most of the strains. The results are shown in table 1. The mice were observed for a week after inoculation. When death occurred it was usually

TABLE 1

Results of injection into mice

SOURCE	FERMENTATIVE GROUP	MICE	
		Survived	Died
Human....	Lactose −, Salicin +	17	61
	Lactose +, Salicin +	15	36
	Lactose +, Salicin −	1	5
Bovine..............	Lactose +, Salicin +	20	5
	Lactose +, Salicin −	16	1
Guinea pig...........	Lactose −, Salicin +	1	2
Rabbit..............	Lactose −, Salicin +		1
Horse...............	Lactose +, Salicin +	1	

within 48 hours. If the inoculation of mice with such large amounts of culture is of any significance, it would appear that strains of the lactose −, salicin + group are more frequently pathogenic for mice than are the other strains. It also appears that the human strains of whatever fermentative group are more frequently pathogenic for mice than are the bovine strains.

When hemolysin tests were carried out by adding 0.5 cc. of a 5-per cent suspension of washed rabbit-blood erythrocytes to 0.5 cc. of young serum broth cultures and incubating for two hours, the double-zone hemolytic streptococci were variable in their reactions, i.e., different strains produced various amounts of hemolysis, from slight to complete. Most strains produced

moderate hemolysis. The amount of hemolysis was independent of the source or fermentation reactions of the strains. These streptococci are therefore less consistently hemolytic by this test than are strains of serological groups A and C.

All of the 188 strains of double-zone beta-hemolytic streptococci studied have been found to belong to Lancefield's serological group B by the precipitin test. Seventy-five of these strains had been grouped by other investigators (Lancefield and Plummer) using the Lancefield technique (1933). Most of them, and all of the remaining strains, were also grouped by us, using the microscopic technique of Brown (1938). We have also encountered a few hemolytic streptococci of bovine origin falling into group B which did not produce double zones in our media but which were otherwise culturally like the double-zone strains. It may be said, therefore, that according to our experience to date, all double-zone beta-hemolytic streptococci belong to serological group B but that there may be a few strains of group B which do not produce double zones under the conditions which we have employed.

Sixty-seven of the double-zone strains have been serologically "typed" by Lancefield or Plummer. No close correlation of source, fermentative characters and serological types was apparent. Further study will be necessary to determine whether there is any correlation.

In table 2 the specific sources and the fermentative groups of all of the double-zone beta-hemolytic streptococci studied are listed. In human beings the throat and the genito-urinary tract may be regarded as their habitat. Of the 22 throats from which strains were isolated, only two were described as "sore" and it is not certain that the condition of these two was due to the double-zone streptococci. Nothing is known of two of the tonsil cases and the other two tonsil strains were recovered from removed tonsils, one patient having complained of frequent sore throat. These were the only strains found in the examination of 100 pairs of removed tonsils. Again, the relation of the double-zone streptococcus to the clinical condition is unknown. Most of the 46 vaginal strains were taken post-partum from women with

afebrile puerperium.   One was from a woman with a post-partum temperature of 100°F. but without other evidence of infection reported.   A few strains were found ante-partum during routine examinations.   Of the 32 urine cultures all were from cases of

TABLE 2

Sources and fermentative groups of human strains studied

SOURCE (HUMAN)	LACTOSE—SALICIN+	LACTOSE+SALICIN+	LACTOSE+SALICIN—
Throat..................................	7	9	6*
Tonsils..................................	2	2	
Vagina..................................	20	26	
Urine..................................	26	6	
(Probably derived from the mouth or throat)			
Sinus..............................	1		
Lung...	10	4	
Heart blood, 15116....................	1		
Neck abscess.........................		1	
(Probably derived from the genito-urinary tract)			
Pelvic abscess.................	2		
Blood (abortion) during life...........		1	
Knee..................................	1		
Heart blood, post-mortem.............	6		
Peritoneum...........................	2	1	
(Infections of obscure origin)			
Peritoneum...........................		1	
Ventricular fluid (hydrocephalis)......	1		
Gangrenous leg.......................	1		
Source unknown (Lancefield 090)........		1	

* These six strains were obtained from Plummer and are representative of those found by her in the throats of children in Institution B.

urinary tract infection (cystitis, pyelitis, urethritis, prostatitis). Three-fourths of them were from women.   In sixteen instances no other organism than the double-zone beta-hemolytic streptococcus was obtained in culture and the latter was often present in large numbers.   There seems no doubt that these streptococci

may be a cause of such infections of the urinary tract. It is to be noted that most of the strains recovered from urine failed to ferment lactose.

Hare and Maxted (1935) were unable to find hemolytic streptococci of serological group B in the stools of 150 women examined. Smith and Sherman (1938) reported six strains isolated from human feces. The normal throat and the vagina of some individuals appear to be the habitats of double-zone beta-hemolytic streptococci. From these sources infection may occur, as indicated in table 2. Some of the cases may be worthy of discussion.

Fourteen strains were obtained from lungs at autopsy but always mixed with other organisms. The pathogenic rôle of the double-zone streptococci is doubtful in these cases.

Case 15116 was one of typhoid fever of several weeks duration (male, age 36). At autopsy there was no perforation of the intestine and no peritonitis but there were found purulent sinusitis, otitis media and a deep ulceration of the vocal cord extended into the cartilage. At the base of the ulcer were masses of Gram + cocci. From the heart blood at autopsy there were obtained large numbers of double-zone hemolytic streptococci and a smaller number of colon bacilli. It seems probable that death was due to terminal blood stream infection by double-zone streptococci derived from the throat.

The pelvic abscesses were in women and in one of them the double-zone streptococcus was found in pure culture.

In the fatal case of septicemia following abortion 100 double-zone hemolytic streptococci per cubic centimeter of blood were found in pure culture during life.

From an arthritis of the knee, double-zone hemolytic streptococci in large numbers were found in pure culture. A similar organism was found in small numbers in the blood stream. There had been a chronic pyuria and the patient was diabetic. Death followed operation on the knee but the cause of death was not determined.

In six other cases double-zone hemolytic streptococci were cultured from the heart blood post-mortem. Three of the cases were infants in the blood of whom only the streptococci were found; one lived only 24 hours after birth, one four days with hemorrhagic disease of the new-born, and one was born prematurely but lived for one month. Infection may have been contracted from the mothers at birth. The

other three cases were adults with pathological findings which suggest possible infection from the genito-urinary tract (carcinoma of the bladder, pyelonephritis and cystitis, arsphenamine poisoning with salpingitis and cysts in the ovaries and cervix uteri).

In three cases peritoneal infection appeared to be correlated with genito-urinary lesions (abscess of the broad ligament, carcinoma of the prostate and adenoma of the kidney, pyelonephritis and cystitis).

In three cases the infections were of obscure origin and in the ventricular fluid of one of them the double-zone streptococci were found in pure culture.

Reference to table 3 shows that 80 of the strains isolated from human beings were of the lactose — group and that 18 of the

TABLE 3

Strains from all sources

SOURCE	LACTOSE− SALICIN+	LACTOSE+ SALICIN+	LACTOSE+ SALICIN−
Human........................	80	52	6*
Bovine........................		27	18
Guinea pig	3		
Rabbit........................	1		
Horse.........................		1	
Totals.....................	84	80	24

* These six strains were obtained from Plummer and are representative of those found by her in the throats of children in Institution B.

strains from cow's milk were of the salicin — group.   To date, lactose — strains have not been reported from bovine sources and salicin — strains have not been reported from pathological conditions in man.   It is obvious that the human infant may have an opportunity to acquire double-zone hemolytic streptococci at birth.   There certainly is no evidence that the lactose — strains found in man are derived from cows milk.   Judging by their history (Plummer, 1934) the 6 salicin — strains isolated from children's throats may have been of bovine origin.   If the human throat may serve as a carrier of salicin — strains derived from milk, there appears no reason why it may not also carry lactose +, salicin + strains from milk, but it does not follow that all such strains are

from milk. Strains fermenting both lactose and salicin were from both human and bovine sources and appear to be alike by current methods of study, but to speculate as to whether such strains found in man were originally bovine seems as fruitless as would be an effort to determine whether the colon bacilli originated in the intestinal tract of the cow or man. The pathogenic rôle of these streptococci for man bears a certain similarity to that of the colon bacilli. In the blood stream they may produce septicemia; in the abdomen, peritonitis; in the urinary tract, cystitis or pyelitis; they may infect sinuses or produce localized abscesses in various parts of the body. The invasion of the blood stream by double-zone hemolytic streptococci is not always fatal. We have on record a case of puerperal sepsis (retained placenta) in which the streptococci were found in the blood for nearly two months, followed by recovery. The prognosis for infections with these streptococci is more favorable than for infections with group A streptococci. The more than sixty strains in our collection from human autopsies and pathological conditions gives undue prominence to their pathogenicity. From 2000 autopsies cultured in this laboratory there were isolated 307 strains of beta-hemolytic streptococci and only 45 of these were double-zone streptococci. Very few of the latter could be suspected as the cause of death, and in many instances they were of no greater significance than other terminal or postmortem invaders. In other than a hospital population one might collect a large number of such strains without encountering pathological conditions. These streptococci are often harbored by normal individuals. They appear to be opportunist pathogens, as are many other organisms normally harbored by man, and there is no evidence that they cause contagious disease or are of epidemic significance.

There is evidence that the lactose +, salicin + and the lactose +, salicin − double-zone beta-hemolytic streptococci are of epizootic importance. Of the 45 strains from cow's milk, twenty-seven, including all but one of the salicin − strains, were from individual cows with clinical mastitis; five were received from other laboratories, labeled *Streptococcus mastitidis*; and the re-

maining fourteen were from mixed milk or were simply labeled
"from milk" when received.   Both the lactose +, salicin + and
the lactose +, salicin − strains are causes of mastitis and prob-
ably are commonly transmitted from cow to cow during milking.
R. B. Little (1937a) has shown that experimental mastitis may be
produced with small amounts of culture introduced beyond the
sphincter without injury to the teat, or by allowing the teat to
aspirate culture or contaminated milk from a dish held in the
hand (1937b).   By the former method, Little (1938) produced
mastitis in first-calf heifers with six double-zone beta-hemolytic
streptococci isolated by us from human sources.   See table 4.

In view of the above results there must be entertained the
possibility of the infection of cows with double-zone streptococci

TABLE 4

Strains used for inoculation of cows udders

STRAIN	SOURCE	SEROLOGICAL		FERMENTATION	
		Group	Type	Lactose	Salicin
Nr.........	Septicemia following abortion	B	III	+	+
Fy.	Normal throat	B	II	+	+
T78.......	Removed tonsil	B	Unclassified	+	+
BB.........	Blood of new-born infant	B	Ib	−	+
Gn.........	Normal throat	B	Ib	−	+
Py.........	Urine: cystitis	B	III	−	+

of human origin by the hands of milkers.   That it may not
happen frequently is indicated by the relative infrequency of the
infection of the udder by group A hemolytic streptococci and by
the absence, to date, of reports of the isolation of lactose −, group
B hemolytic streptococci from the udder.   It would appear that
the human carrier needs to be considered, in attempts to eradicate
mastitis from herds of cattle.

Among the double-zone beta-hemolytic streptococci studied
were five from other animals than cows (table 3).   Three strains
were from guinea pigs, two from infected eyes and one from a
foot, reported by Parsons and Hyde (1928).   One strain was
from a rabbit (Lancefield's strain K 158 A) used for testing
vaccine virus.   The above four strains were lactose −, salicin +.

The fifth strain was lactose +, salicin + and was isolated in 1908 from a horse with the diagnosis of "strangles." The stock strain has not been subjected to animal passage since its isolation. No other details of the case are known.

The lactose + double-zone beta-hemolytic streptococci would fall into "Group Ib" mastitis streptococci as described by Minett (1935), the *Streptococcus agalactiae* of some authors and the *Streptococcus mastitidis* of others. The tendency of some is to recognize both hemolytic and non-hemolytic varieties under these species, since both are common causes of mastitis and some of the non-hemolytic strains are reported to belong to serological group B and to be similar to the hemolytic strains except with regard to hemolysis. However, only a limited number of cultural tests have been employed and the discovery of other tests may reveal significant differences as was the case with *Streptococcus pyogenes* and some of the animal strains of serological group C previously indistinguishable from *Streptococcus pyogenes*. Frost, Gumm and Thomas (1927) proposed the name *Streptococcus asalignus* for the salicin − hemolytic streptococcus from milk which probably is identical with our lactose +, salicin − double-zone hemolytic streptococcus. Frost and Engelbrecht (in press) propose the name *Streptococcus mastitidis* for the hemolytic lactose +, salicin + mastitis streptococcus and the name *Streptococcus agalactiae* for the otherwise similar non-hemolytic mastitis streptococcus. There is equally good reason for giving a specific name to the lactose −, salicin + double-zone beta-hemolytic streptococcus belonging to serological group B. It would be a convenience to be able to designate these cultural entities by specific names, since the use of more general terms, the letters of the alphabet and even the Roman and Arabic numerals is becoming confusing and is meaningless without reference to the individual author cited. However, this is a situation which probably should be tolerated until more is known about the meaning of bacterial species in a rapidly developing field of study. The inclusion of more than one species in a single serological group need be no deterrent, since most authors recognize several species of hemolytic streptococci in serological groups A, C and

D. Neither need the finding of similar type antigens in bacteria of different species or genera be disturbing (recently discussed by Lancefield, 1938). It would appear that a species of *Streptococcus* need not be limited to a single serological type but should not transcend a serological group. I question whether streptococci of the same serological group and type need be placed in the same species if they show stable cultural differences.

No instability nor variability has been noted in the strains of double-zone beta-hemolytic streptococci in our possession although some of them have been under observation for twenty-five years.

SUMMARY AND CONCLUSIONS

Double-zone beta-hemolytic streptococci are found in the throats and vaginae of many normal persons. They are also a common cause of mastitis in cows.

These streptococci fall into three fermentative groups: lactose −, salicin +; lactose +, salicin +; and lactose +, salicin −.

All of the 188 strains examined belong to Lancefield's serological group B by the precipitin test.

In man, the lactose −, salicin + and the lactose +, salicin + strains may assume the rôle of opportunist pathogens, producing infections in various parts of the body and rarely may cause fatal septicemia. The source of such infections may usually be traced to the throat or genito-urinary tract. There is no evidence that these streptococci are of epidemic significance.

In cows, the lactose +, salicin + and the lactose +, salicin − strains are common causes of mastitis. The lactose −, salicin + strains have not been found in cows although they are capable of producing mastitis when inoculated into the udder.

In attempts to eradicate mastitis from dairy herds the human carrier of these streptococci should be considered.

The recognition of the double-zone hemolytic streptooocci supplies additional reason why diagnostic laboratories should no longer report all hemolytic streptococci as *Streptococcus hemolyticus,* a term which is inadequately informative and of doubtful validity as a specific name.

The author is grateful to those who have supplied cultures for this study, especially to Dr. Rebecca C. Lancefield, not only for many cultures but also for the serological grouping and typing of strains submitted to her.

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PHYSIOLOGICAL YOUTH AS AN IMPORTANT FACTOR IN ADAPTIVE ENZYME FORMATION

C. P. HEGARTY[1]

Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca, New York

It has been known for many years that bacteria, cultivated upon a specific substrate, may not ferment other materials unless cell multiplication occurs. Karström (1930) observed that cells grown in the presence of one sugar, then suspended in solutions of other sugars under conditions preventing multiplication, could not ferment the new sugars for long periods of time, if at all. Stephenson and Strickland (1933) showed that there was no natural selection in the case of formate adaptation by *Escherichia coli* and that the formation of the enzyme can occur while no cell division is taking place. Stephenson and Yudkin (1936) observed that top yeasts can adaptively ferment galactose without cell multiplication but no adaptation could be obtained with cultures incapable of growth. They state, however, that a study of their total and viable counts suggests that it is not the viable cells alone which are capable of adaptation. Stephenson and Gale (1937) found that the galactozymase of *Escherichia coli* was formed only when growth occurred in the presence of the specific substrate. This adaptation was proportional to the number of cells which had divided in the presence of the galactose.

The following experiments are the result of a search for the factors controlling the mechanism of adaptation. Old cells adapt themselves slowly if at all (Karström 1930) and young, growing cultures produce the new enzyme easily. Therefore, experiments were designed to test cells of various ages and to determine

[1] Now with the Department of Bacteriology, Oregon State College, Corvallis, Oregon.

the stage of growth during which new enzymes are most easily manufactured.

<div style="text-align:center">METHODS</div>

All of the experiments were performed with a culture of *Streptococcus lactis* (No. 125) which ferments all of the sugars characteristic of that organism when grown in nutrient media containing the various carbohydrates. The cells were grown in a medium consisting of 0.5 per cent peptone, 0.5 per cent tryptone, 1.5 per cent phosphate buffer at pH 7.0 and 0.5 per cent of the chosen carbohydrate (Rahn, Hegarty and Deuel, 1938). When the culture was at the desired age, the cells were centrifuged and resuspended in a solution containing 0.5 per cent peptone, 2.0 per cent phosphate buffer at pH 7.0 and 2.0 per cent of the carbohydrate to be tested. A heavy suspension of cells was used. With full-grown cultures, the cells from 250 cc. of medium were resuspended in 50 cc. of a 2 per cent buffer solution, and with growing cultures the ratio was changed accordingly. Frequent plate counts and Petroff-Hausser counts (Knaysi, 1935) showed no indications of growth in any of these suspensions. The rate of fermentation was determined by titration of 5.0 cc. samples with N/10 NaOH. If the cells were difficult to obtain, 1.0 cc. samples were titrated with N/18 NaOH, using a micro-burette.

Preliminary experiments showed that the enzymes attacking glucose, fructose and mannose were constitutive, i.e., produced under all circumstances, while the enzymes attacking all of the other sugars were adaptive, according to the terminology of Karström (1930).

A 12-hour old culture in glucose medium was centrifuged, and the cells resuspended in the buffer solution. One-third of this contained 2 per cent glucose, one-third 2 per cent sucrose, and one-third 2 per cent sucrose + 0.2 per cent glucose. Glucose was fermented from the start. No measurable acidity was produced from sucrose within 5 hours. The cells in sucrose + 0.2 per cent glucose produced 0.18 per cent lactic acid within the first hour, utilizing the glucose, but no further fermentation occurred until after 5 hours. The initiation of fermentation of one sugar had no effect upon acid production from other sugars.

A series of experiments was designed to determine the effect of the age of the culture upon adaptation.   The cells were grown in glucose-tryptone broth, centrifuged .out after various periods of incubation, and placed into buffered solutions of glucose and sucrose.   Table 1 shows the gradual development of lactic acid from glucose and sucrose by cells centrifuged at various ages of the culture.   All of the cells, regardless of age of the culture, fermented glucose from the start at rapid rates.   The cells centrifuged when the culture was 2 hours old started fermentation in the sucrose medium earlier than cells obtained when the

TABLE 1

Per cent lactic acid formed from glucose and sucrose by glucose-grown cells of various ages

AGE OF CELLS	SUGAR	TIME IN HOURS					
		1	2	3	4	5	6
hours							
2	Glucose	.45	.50	.57	.57	.58	.58
2	Sucrose	.00	.05	.09	.14	.16	.19
12	Glucose	.23	.38	.41	.45	.47	.48
12	Sucrose	.00	.00	.00	.03	.03	.03
24	Glucose	.09	.22	.31	.43	.51	
24	Sucrose	.00	.00	.00	.03	.04	.04
48	Glucose	.04	.10	.14	.21	.26	
48	Sucrose	.00	.00	.00	.00	.01	.01

culture was older and adaptation required more time as the age of the culture increased.

A more extensive study was made of adaptation to galactose. 13.5 liters of glucose-tryptone broth were inoculated with 1.5 l. of a 36-hour culture of *Streptococcus lactis*.   At the start and after every hour, samples of this culture were centrifuged and the cells resuspended in buffer plus glucose or galactose.   The curves of these fermentations are shown in figure 1.   Glucose was fermented from the start by cells of all ages.   The number of cells per cubic centimeter of suspension could not be kept the same.   The suspensions from the 3- and 4-hour old cultures contained about twice as many organisms per cubic centimeter as the 1- and 2-hour suspensions.   The fermenting capacity per cell

per hour was 14.5×10^{-10} mgm. glucose for the 1 hour cells and had dropped to 9.3×10^{-10} mgm. when the culture was 4 hours old. These facts account for the differences in the rate of glucose fermentation.

Quite different are the fermentation curves for galactose. The cells collected when the culture was 1 hour old started fermentation soon after resuspension while the 4-hour cells required 9 hours for adaptation. Cells collected after longer periods of

ADAPTATION TO GALACTOSE BY GLUCOSE-GROWN CELLS
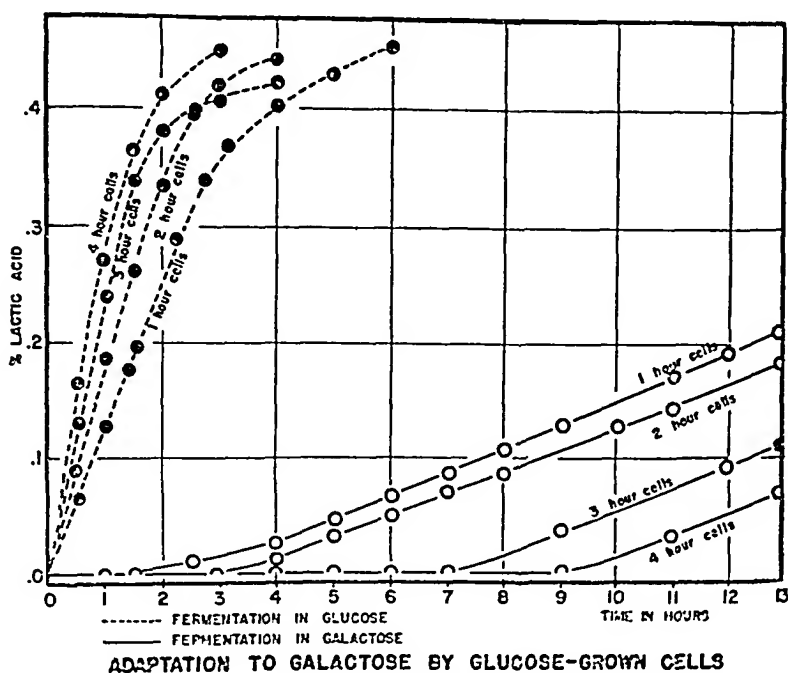
Fig. 1

incubation (not shown on the graph) did not produce any acid for 13 hours. The growth curve of the original culture supplying the cells for this experiment (fig. 2) showed that after 1 hour, the cells were just coming out of the lag phase. It is evident that as the culture passes from the stage of physiological youth into the logarithmic phase, adaptation becomes slower. Plate counts and Petroff-Hauser counts were made of all suspensions at frequent intervals and no increases in the numbers of cells could be detected.

Two other sets of experiments were performed in the same way, but with different sugars. In one, the cells were grown in glucose, and resuspended in buffer plus glucose, galactose, lactose, or sucrose. In the other experiment, the cells were grown in maltose, and tested in glucose, maltose, and sucrose. In general, the results were the same as those obtained before, as may be seen from table 2. Glucose-grown cells fermented glucose from the start. They showed rapid adaptation to galactose when

GROWTH CURVE OF CULTURE SUPPLYING GLUCOSE-GROWN CELLS
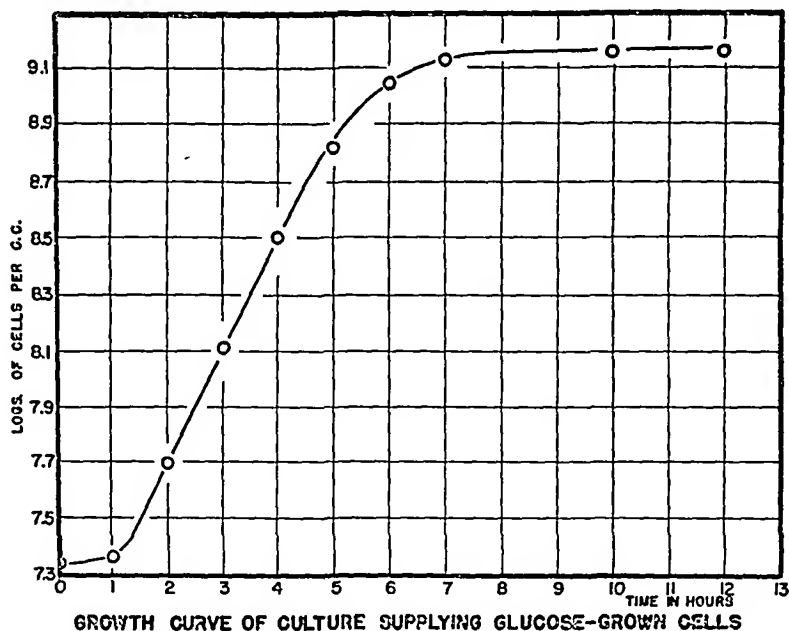
Fig. 2

obtained from a physiologically young culture, but required long initiation times when the culture was older. In lactose, the delay was 8 hours, with cells obtained from a physiologically young culture, and 20 hours, with cells from older cultures. The glucose-grown cells in sucrose showed no delay when the culture was just coming out of the lag phase. A growth curve showed that this culture had a 2-hour lag phase and therefore the cells centrifuged after 2 and 3 hours were physiologically young. The cells from older cultures exhibited a short delay.

In another experiment there was an adaptation time of 5.5 hours before glucose-grown cells produced acid from maltose.

The maltose-grown cells from cultures of all ages showed no delay in either glucose or maltose.   This is not unexpected as the organisms had produced the adaptive maltase during growth in the original maltose culture, and the glucose enzyme is constitutive.   Maltose-grown cells in sucrose required a short adaptation period when old, but showed no delay during physiological youth.   It is interesting that maltose-grown cells from older cultures initiated fermentation in sucrose more rapidly than similar cells grown in glucose.

TABLE 2

Hours delay before adaptation occurs by cells of various ages grown in glucose and maltose

	GROWN IN GLUCOSE				GROWN IN MALTOSE		
AGE OF CELLS	Hours required for adaptation when placed in						
	Glucose	Galactose	Lactose	Sucrose	Glucose	Maltose	Sucrose
36	0	18	29	8	0	0	1.5
1	0	1.5	8	0.5	0	0	0
2	0	3	9	0	0	0	0
3	0	7	15	0	0	0	0
4	0	9	16	1.5	0	0	0
6	0	13	20	3	0	0	0.5
8	0	13	20	4	0	0	0.75
10	0	13	20	4	0	0	0.75

In every case where it was necessary for the organisms to adapt themselves to a new carbohydrate, the cells obtained from physiologically young cultures accomplished this much more rapidly than cells from a later stage of growth.

DISCUSSION

These experiments have shown that at the end of the lag phase, before the maximal growth rate or the logarithmic phase is reached, the cells are most adaptable to new types of food. During the logarithmic phase, while the rate of growth remains constant, adaptability decreases rapidly.

At this stage of early development, the cells are quite different from cells of slightly older cultures as was first shown by Sherman and Albus (1923) who termed them physiologically young. Later Bayne-Jones and Rhees (1928), Walker, Winslow, Huntington and Mooney (1934), Mooney and Winslow (1935) Huntington and Winslow (1937) and others have shown that the rate of metabolism per cell (production of heat, CO_2, NH_3, oxygen consumption, etc.) is maximal just before the logarithmic growth rate is reached.

Hershey and Bronfenbrenner (1938) state that "correlations of viable counts, centrifugeable nitrogen and turbidity, with oxygen consumption, indicate that the increased metabolism during the early portion of the growth period is quantitatively referable to increased average size of cell," which is not in complete agreement with the work of Huntington and Winslow (1937). While all these properties represent only quantitative changes, the case of adaptive enzymes is also a qualitative one, and therefore more significant, and in a class by itself.

It seems quite noteworthy that at the stage of rejuvenation of the old cells (Sherman and Albus, 1924), when the cell shows the most intensive metabolic activity, with distinct morphological changes (Henrici, 1928), the cell is also most ready for physiological changes and adaptations.

CONCLUSIONS

Cells from a mature culture of *Streptococcus lactis* in glucose-tryptone broth can not attack galactose, lactose, sucrose or maltose at once, unless they have been allowed to multiply in the presence of that sugar.

If cells are obtained from a glucose culture while still in the stage of physiological youth, they may attack these sugars either at once or with a short delay, even under conditions preventing proliferation.

The most rapid adaptation to new sugars is always observed with cells from cultures just coming out of the lag phase, during the period of physiological youth.   During the logarithmic phase, adaptability decreases rapidly and continuously.

The ease of adaptation and the rate of loss of adaptability during the ageing of a culture varies greatly with each sugar.

The author expresses his appreciation to Dr. Otto Rahn for the suggestions and assistance given in the experimental work and in the preparation of the manuscript.

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A METHOD FOR PRODUCING INCREASED CARBON DIOXIDE TENSION IN INDIVIDUAL CULTURE TUBES AND FLASKS

H. J. SHAUGHNESSY

Department of Bacteriology and Public Health, University of Colorado School of Medicine and Hospitals, Denver, Colorado[1]

During the past few years there has been a growing appreciation of the rôle of carbon dioxide in bacteriology. It has been demonstrated by a number of observers that the presence of carbon dioxide is apparently necessary for growth of all species of bacteria so far studied. Walker (1932) and Gladstone *et al.* (1935) suggest that the lag phase of the bacterial growth cycle is due to the failure of bacteria to multiply until a certain minimum amount of carbon dioxide is produced in the culture by their activities. If this is correct, it would appear that carbon dioxide should be supplied to cultures of feebly-growing organisms and to cultures in which the inoculum is small such as, for example, single cell cultures. Since the size of the inoculum, in terms of the number of bacterial cells, is not usually known when cultures are made from exudates, blood or other materials from suspected cases of disease, it follows that the routine provision of increased carbon dioxide tensions in such cultures would be desirable.

The use of increased carbon dioxide tensions has been recommended, especially for isolating gonococci, meningococci and *Brucella abortus*. A number of investigators, including McLeod and his associates (1934), Leahy and Carpenter (1936) and Thompson (1935) have demonstrated the value of incubating cultures for the isolation of gonococci from suspected cases in an atmosphere containing about 10 per cent CO_2. Cohen and Flem-

[1] The author is now located in the Division of Laboratories, Illinois Department of Public Health, 1800 West Fillmore St., Chicago.

ing (1918) as early as 1918 found the same percentage of CO_2 to be optimum for the isolation of meningococci from spinal fluids and nasopharyngeal cultures, a conclusion recently confirmed by Thompson (1935). The investigations of Huddleson (1921), Smith (1924), McAlpine and Slanetz (1928) and Wilson (1931) have shown that most strains of *B. abortus* grow best under a CO_2-tension of 5 to 10 per cent. The use of an increased tension of this gas in the primary isolation of bacteria of the *B. abortus* group has become routine in some laboratories in which appreciable numbers of cultures for the isolation of these bacteria are being made.

Increased carbon dioxide tension has found another important use in connection with the production of the exotoxins of some bacteria. Burnet (1930) introduced into common use the method of growing staphylococci in a semi-solid medium in an atmosphere containing about 20 per cent CO_2 in order to produce potent exotoxins. It has been found (Woolpert and Dack, 1933) also that staphylococcus enterotoxin, which is apparently distinct from the other exotoxins of staphylococci, can be produced more consistently in the presence of 20 to 30 per cent CO_2 than in the unaltered atmosphere. More recently enterotoxins have been obtained from streptococci, *Proteus*, *Salmonella aertrycke* and *Escherichia coli* by this method (Jordan and Burrows, 1935; Cary, Dack and Davison, 1938).

In spite of the demonstrated value of using increased carbon dioxide tensions in many phases of bacteriology, this procedure is not used routinely in most laboratories, even in cultures where its use is practically a requisite for success. This is due probably to the fact that it is cumbersome to use jars in which to incubate the cultures. Thompson's method (1935) of producing CO_2 in jars has simplified their use greatly. When culture jars are used, however, it is usually necessary to open the jars to see whether growth has occurred in the individual tubes, flasks or Petri dishes. If further incubation is necessary, the whole procedure of filling the jar with carbon dioxide must be repeated.

The method mentioned by Parker and Hudson (1926) for producing increased CO_2 tensions by burning a candle in the jar

in which the cultures are incubated is of distinct but limited value. CO_2 concentrations of about 3 per cent are apparently the maxima which can be produced by this technique (Nye and Lamb, 1936).   This percentage is inadequate for the best results (McLeod et al., 1934; Cohen and Fleming, 1918; McAlpine and Slanetz, 1928; Burnet, 1930).   The use of the jar in this method is also subject to the criticism mentioned above.

Joyner and Jones (1937) have described a method recently by which the Spray (1930) anaerobic culture dish can be used for incubating individual cultures in an increased carbon dioxide tension.   While this appears to be a valuable method, at least four criticisms may be levelled against it.   Spray plates are relatively expensive.   It is not possible, of course, to use liquid or semi-solid media in them which precludes their use for toxin production, for example.   It is difficult to observe colonies on an opaque medium such as the "chocolate" agar commonly used in culturing gonococci without breaking the seal and remaking the whole culture.   The most serious objection to the Spray plate method is that special culture media must be kept prepared in the plates even though cultures under CO_2 tension are only occasionally made in a laboratory.

The author has sought a simple method that can be applied to individual culture tubes and flasks, free from the objections mentioned above.   It seems especially desirable to develop a method by which liquid media used for toxin production can be incubated in individual flasks.   It also appeared that the method should allow the bacteriologist to utilize any type of medium desired for a particular purpose without making it necessary to place the medium in a special container beforehand.

Hall's (1937) modification of the method devised by Wright which is used routinely in this laboratory for absorbing oxygen from culture tubes was first tried.   It was found, however, that, if the proper amount of bicarbonate to produce a certain CO_2 tension was placed on the cotton stopper, a great deal of the gas was lost before the rubber stopper could be inserted.   In order to obviate this difficulty the following technique was evolved. The upper fluffy portion of the sterile cotton stopper of a test

tube or Florence flask is cut off and the remainder pushed as far down into the test tube or neck of the flask as possible without touching the medium. A short glass tube or shell vial of the proper size (about 10 x 35 to 40 mm. for the ordinary 16 to 18 x 150 mm. culture tube) is placed open end upward on the stopper. For flasks it is desirable to use larger vials, their size depending on the size of the flask. A gelatin capsule containing a measured amount of bicarbonate solution is placed in the inner tube or vial. A number 0 capsule has been found satisfactory for the test tube cultures but larger or smaller capsules may, of course, be used depending upon the amount of bicarbonate solution required for a particular purpose. A sufficient amount of sulphuric acid to cover the capsule, about 1 cc. for the test tube culture, is then placed in the vial and the whole culture tube sealed with a closely fitting rubber stopper. After about five minutes at room temperature the gelatin capsule begins to disintegrate and carbon dioxide begins to evolve gently. The method, which is illustrated by the diagram in figure 1, is much simpler than the description indicates and only a few seconds are required to make a culture.

Sulphuric acid with a bicarbonate is used as the source of carbon dioxide. Potassium bicarbonate is preferable to the sodium salt because of its greater solubility. To produce a 30 per cent CO_2 tension in a culture tube 16 to 18 x 150 mm., containing about 20 cc. of atmosphere above the medium, about 6 cc. of CO_2 are needed. At 20°C., assuming normal pressure, approximately 7.2 cc. of CO_2 are liberated from 0.1 cc. of three-molar potassium bicarbonate solution. For liberating the gas it is convenient to use an excess of acid so that it is unnecessary to measure it. Since it is desirable to provide sufficient acid to cover the gelatin capsule, we have found that 1 cc. of sulphuric acid diluted 1:30 as recommended by Thompson (1935) is suitable for a test tube culture. For a flask culture an appropriately larger amount of acid should be used.

Tests were made to be certain that the theoretical yield of CO_2 was liberated and diffused throughout the tube under the conditions described above. The atmosphere in the lower portion of

the culture tube, directly over the culture medium, was collected through a side arm and analyzed in a Yandell Henderson (Henderson and Greenberg, 1931) syringe gas analyzer, which has an accuracy sufficient for this purpose.   When the pinchcock between the culture tube and gas analyzer was opened, the plunger in the syringe was forced out by almost exactly the number of cubic centimeters that should have been yielded theoretically
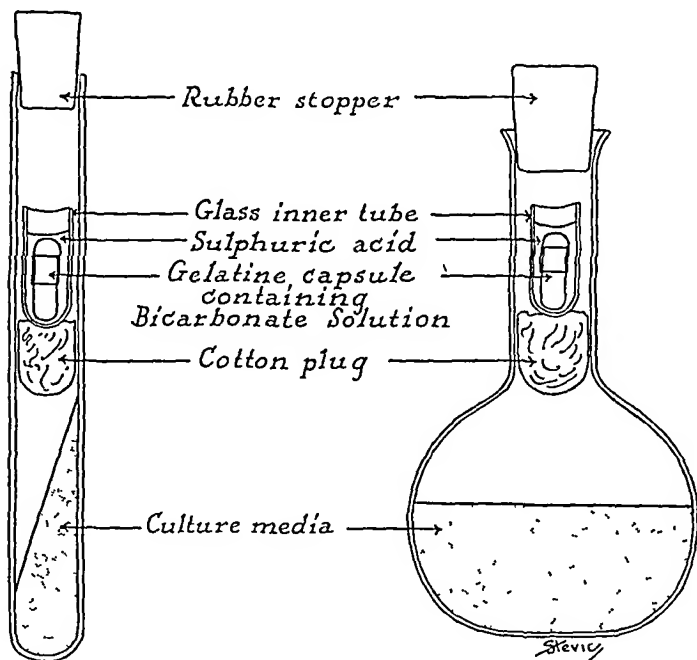
FIG. 1. SCHEMATIC DRAWING ILLUSTRATING METHOD

from the mixtures of bicarbonate and acid used.   There were also very good approximations between the theoretical percentages of CO_2 which should have been present and the actual percentages found by gas analysis.

It has also been found that this method may be applied to anaerobic cultures.   In this case, pyrogallic acid is placed in the inner vial, either directly or in a gelatin capsule, and 10 per cent sodium hydroxide is run in over it.   The amounts should be

adjusted so that good absorption of oxygen takes place. About 1.5 grams of resublimed pyrogallic acid and 2.0 cc. of 10 per cent sodium hydroxide are satisfactory for the usual culture tube. An excess of pyrogallic acid should be maintained, of course, to prevent absorption of CO_2 by the alkali. In this connection the interesting possibility arises of using combined oxygen absorption and increased CO_2 tension to promote the growth of anaerobic or microaerophilic bacteria.

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GROWTH FACTOR REQUIREMENTS OF THE ROOT NODULE BACTERIA[1]

P. M. WEST AND P. W. WILSON

College of Agriculture, University of Wisconsin, Madison

It is known that yeast or plant extracts markedly stimulate the growth of the root nodule organism in media of purified ingredients. An essential growth factor or superior source of nitrogen has been invoked to explain the results, but neither theory has a broad experimental basis. The results of Allyn and Baldwin (1930) suggest that the extracts may affect favorably the oxidation-reduction potential of the medium. Allison, Hoover and Burk (1933) report that a specific co-enzyme for respiration of rhizobia can be extracted from commercial sucrose, azotobacter cultures and various plant or animal tissues. This extract markedly stimulates the growth of rhizobia in a medium of purified ingredients. Hoover and Allison (1935) state, "the growth of certain species, on the usual synthetic (sugar, inorganic salts, and nitrate) medium is negligible if pure ingredients are used, and that the addition of a small amount of growth factor is essential."

Thorne and Walker (1936) also found that an extract of azotobacter (or other substances) was able to stimulate growth of several species of rhizobia in a medium of highly purified materials but did not regard it as essential for growth. By substituting ammonium chloride or asparagin for potassium nitrate, they grew four species in repeated transfer for more than two months with no appreciable decrease in rate of growth of the organisms. Addition of reducing substances to Allison and

Hoover's synthetic medium improved it, indicating that the potassium nitrate in this medium poised it at an oxidation-reduction potential too high for continued growth.

Clark (1936) was able to secure only very slight or no growth in the absence of added organic stimulants. Nilsson, Bjälfve and Burström (1938a) observed no growth in the synthetic medium of Allison and Hoover even if NH₄Cl or asparagine were used as the source of nitrogen. Likewise, the addition of reducing substances failed to favor growth; normal growth occurred only in the presence of yeast extract.

In view of the contradictory findings, it seemed desirable to investigate further the claims that certain unknown substances are necessary for growth of rhizobia, and to determine what factors are important for successful continuous transfer of the organisms in a synthetic medium.

EXPERIMENTAL

To avoid confusion in interpretation of results, the base medium adopted was that of Allison and Hoover (1934), consisting of mineral salts, one per cent mannitol, and 200 parts per million nitrogen as potassium nitrate. *Rhizobium trifolii*, Wisconsin strain 205 was used as the test organism.

The "coenzyme R" preparations employed were made from cultures of *Azotobacter vinelandii* according to Hoover and Allison's procedure (1935). In preliminary work a Petroff-Hauser direct count was made on fluid cultures of the organism to determine growth. Later, the growth response was measured by turbidity of fluid cultures as indicated by the Evelyn electrophotometer or development of giant colonies on agar.

Factors influencing rate of growth of R. trifolii in base medium

1. *Yeast extract and azotobacter extract.* A water extract of yeast (Fred, Baldwin and McCoy, 1932) and Allison's azotobacter extract were selected as representative of the numerous natural preparations known to stimulate the growth of *R. trifolii*. Tubes containing from 5 to 500 parts per million of these extracts were inoculated with a standard loop (approximately 500,000 cells per 10 ml. tube) from a 24-hour 10-per-cent yeast water

culture.   The maximum stimulation occurred in the presence of 25 to 50 parts per million of either substance (table 1).   It was noted that azotobacter extract consistently failed to replace, completely, yeast extract in fluid culture (fig. 1), while both were equally stimulative to growth on agar (Plate 1).   In fluid culture, growth in the base medium was, in all cases, relatively slight, and failed completely after three or four transfers.   From these data alone, one might conclude that certain stimulative organic enrichments are required for the prolonged growth of the organism in laboratory media.   However, subsequent experiments demonstrate the need for modification of this opinion.

TABLE 1

Comparative stimulative effects of "coenzyme R" preparations and yeast extract
Counts in millions per ml.

MEDIA	2 DAYS	3 DAYS	4 DAYS
Coenzyme R, 5 ppm....................	92	206	285
Yeast extract, 5 ppm....:..............	160	385	586
Coenzyme R, 10 ppm..................	70	200	308
Yeast extract, 10 ppm..................	318	635	720
Coenzyme R, 25 ppm..................	75	280	341
Yeast extract, 25 ppm..................	485	856	840
Coenzyme R, 50 ppm..................	63	312	322
Yeast extract, 50 ppm..................	580	830	836
Control.............................	<5	15	65

2. *Reducing substances.*   Since the base medium was oxidizing in nature, the possibility of increased growth of the bacteria in the presence of reducing substances was investigated.   Tubes containing sodium sulfite, sodium nitrite and sodium thioglycollate were inoculated from a twenty-four-hour culture as before. Table 2 indicates the amount of growth obtained in cultures supplied with optimal quantities of each compound.   These substances all improved growth in fluid medium, but, as shown in Plate 1, they are without effect on agar.   The use of mass inocula

apparently overcomes, within reasonable limits, effects of alteration in oxidation-reduction potential.
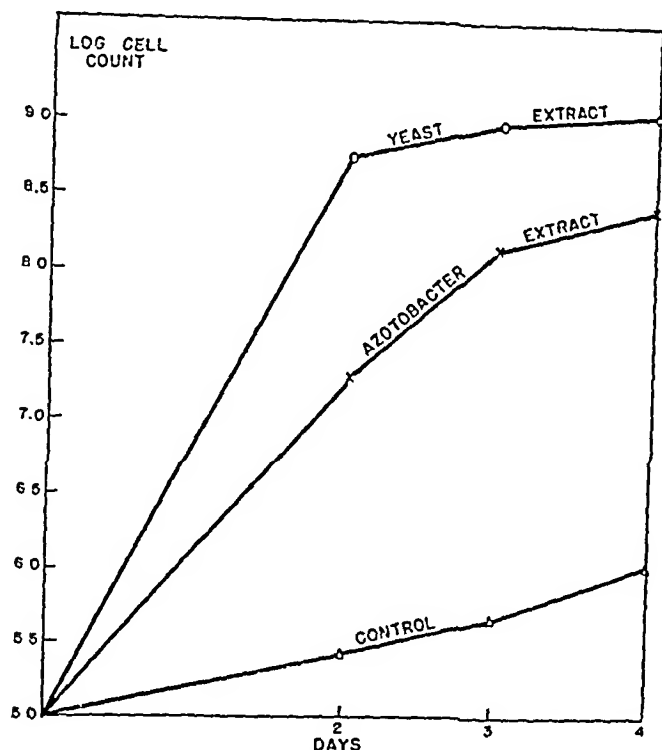
FIG. 1. Comparative stimulative effects of Azotobacter and Yeast extracts (50 ppm.) on growth of *Rhizobium trifolii* in fluid culture.

TABLE 2

Comparative stimulative effects of "coenzyme R" and reducing substances on growth of Rhizobium trifolii

Counts in millions per ml.   Inoculum from 24-hr. culture

MEDIA	2 DAYS	3 DAYS	4 DAYS
Sodium nitrite, 25 ppm.	72	245	318
Sodium sulfite, 25 ppm	75	188	345
Sodium thioglycollate, 10 ppm ..	85	390	446
Coenzyme R, 50 ppm	75	280	341
Control	<5	15	75

Figure 2 shows the relative stimulative abilities of thioglycollic acid and azotobacter extract, both at a concentration of 10 ppm.

At this concentration the stimulation of growth in fluid culture, due to azotobacter extracts, is of the same order as that effected by reducing substances. However, it is clear from Plate 1 that the former contains material of benefit to the organism for reasons other than its ability to alter the oxidation-reduction potential of the medium.
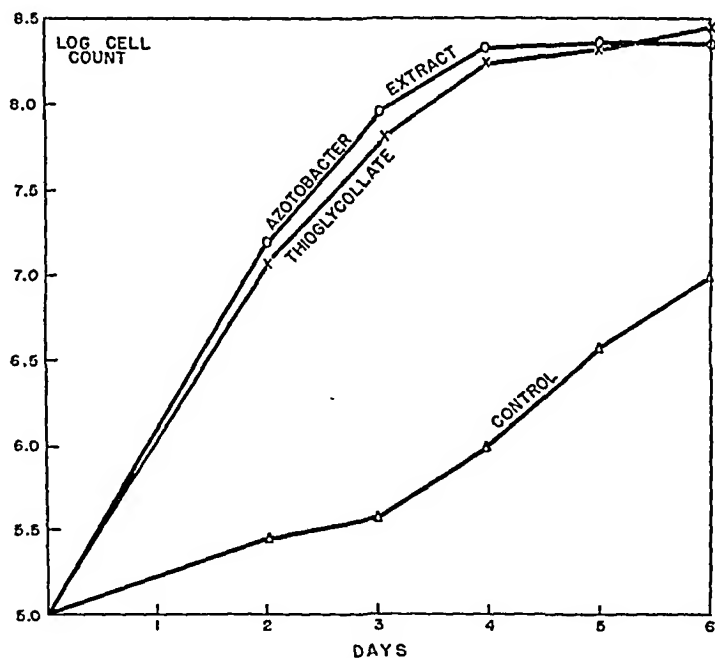
FIG. 2. Comparative stimulative effects of Azotobacter extract and thioglycollic acid (10 ppm.) on growth of *Rhizobium trifolii* in fluid culture.

Continuous growth of Rhizobium trifolii in synthetic media

Results of the foregoing experiments suggested the possibility of growing *R. trifolii* in fluid culture in the entire absence of unknown organic enrichments, over an indefinite period, providing the media were at a favorable oxidation-reduction potential. For the purpose of this experiment, the base medium enriched with 10 ppm. thioglycollic acid was selected. The original inoculation consisted of approximately 500,000 cells from a 24-hour 10-per-cent yeast water culture. Loop transfers were made every three days, and after thirty-four successive

transfers in this medium, the organisms were growing at the same rate as in the first transfer (table 3). After this number of transfers, it was concluded that any objection that growth could be due to activators from the original inoculum, was beyond consideration. Mannitol, which had been continuously extracted for 24 hours with absolute ethyl alcohol to remove any soluble activator, was used in a parallel experiment with identical results. Other reducing substances previously studied behaved similarly to thioglycollate. Purified glucose or sucrose could be substituted for mannitol with essentially the same results. Inoculation of a suitably reduced basal medium from either a yeast-extract enriched culture, or one grown in the absence of

TABLE 3

Growth of Rhizobium trifolii through thirty-four successive transfers in synthetic medium (base medium plus thioglycollic acid—10 ppm.)

Counts in millions per ml.

TRANSFER	2 DAYS	3 DAYS	4 DAYS
Original	86	295	382
1st transfer	64	170	284
2nd transfer	75	292	367
18th transfer.....	94	268	412
34th transfer.........	80	246	395

yeast extract for over thirty transfers, gave the same response. Evidently, then, the small amount of yeast extract carried in the inoculum from a 10-per-cent yeast-water culture does not influence the growth. In the presence of greater amounts of yeast extract, however, there is, as has been previously shown, a considerable stimulation beyond the growth induced by reducing substances.

It is concluded from these data, that *R. trifolii* synthesizes all the organic substances essential for its growth from the simple ingredients of the properly reduced base medium; and that although various tissue extracts are stimulative to growth, they are not required.

Growth factors synthesized by Rhizobium trifolii

Further study suggested that continuous transfer of *R. trifolii* in the base medium, properly reduced by thioglycollate, is dependent upon some highly active material, synthesized by the growing culture and transferred in sufficient amount with a loop inoculum (.005 ml.) to stimulate growth initiation in the new medium. Evidence for this view was the observation that cells removed from the medium in which they had grown, and washed free of their metabolic products, produced much less growth when inoculated into fresh media than did cells not washed. This separation of the organisms from the activators they produce is accomplished either by aseptic centrifugation of a fluid culture, or by suspending growth from the surface of an agar slope in fresh medium. An active culture filtrate, free from cells, can be obtained readily by ultra-filtration.

To determine the effect of culture products on the growth of *R. trifolii*, the following were used as inocula:

(a) A 48-hour culture growing in 10-per-cent yeast extract medium.
(b) A suspension of cells from a 10-per-cent yeast-extract agar slope in fresh 10 per cent yeast extract medium.
(c) A suspension of cells from a slope as in (b) in a sterile filtrate from culture (a).

All inocula were adjusted to contain the same number of cells. The results of this experiment (table 4) indicate that the bacteria during their growth synthesize some highly active material, important in growth initiation, which can be separated from the culture by Berkefeld filtration. That this effect is independent of the presence of yeast extract in the medium is shown in figure 3, which demonstrates rates of growth when the inocula used were:

(a) A growing culture, free of yeast or other extracts for 30 transfers.
(b) Cells from (a) centrifuged and resuspended in the fresh basal medium.

These examples, which are typical of a number of similar tests, illustrate the dependence of the organism on activators synthe-

TABLE 4

Influence of filtrate from growing culture on initiation of growth

Counts in millions per ml.

MEDIUM	INOCULUM	2 DAYS	3 DAYS	4 DAYS
Basal medium + 10 ppm. thioglycollate	A. 48-hour culture,	90	390	415
Basal medium + 15 ppm. thioglycollate	growing in 10 per	55	384	395
Basal medium + 20 ppm. thioglycollate	cent yeast ext. medium	72	311	380
Basal medium + 10 ppm. thioglycollate	B. Suspension of	5	15	75
Basal medium + 15 ppm. thioglycollate	cells in sterile 10	5	5	83
Basal medium + 20 ppm. thioglycollate	per cent yeast ext. medium	5	5	35
Basal medium + 10 ppm. thioglycollate	C. Suspension of	106	390	428
Basal medium + 15 ppm. thioglycollate	cells in sterile	93	371	410
Basal medium + 20 ppm. thioglycollate	filtrate from A.	50	210	325
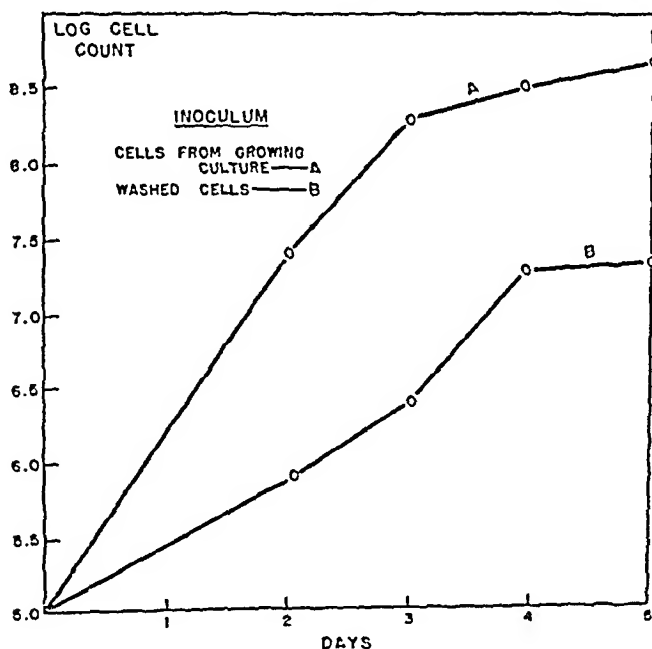
FIG. 3. Effect of type of inoculum on growth of *Rhizobium trifolii* in base medium plus thioglycollate.

sized during the period of active growth of the cells for growth initiation of small inocula.   In the absence of this active material, growth is so slight that continued transfer is impossible.

Influence of heat on growth factor synthesized by R. trifolii

The heat stability of the stimulative factor liberated in growing cultures was determined by subjection of the Berkefeld filtrates obtained therefrom to a temperature of 100°C.   Heating was carried out at neutrality and with the addition of 10 per cent $N/1$ sodium hydroxide or hydrochloric acid.   After heating for periods of 30 minutes and one hour, the tubes were immediately cooled and adjusted to neutrality with sterile acid

TABLE 5

Influence of heat on stimulative properties of filtrates from growing cultures

Counts in millions per ml.

MEDIUM USED FOR SUSPENDING INOCULUM	2 DAYS	3 DAYS	4 DAYS
Filtrate untreated..............................	105	340	385
Filtrate heated at pH 6.8, 30 min..................	<5	20	74
Filtrate heated at pH 6.8, 60 min..................	<5	17	65
Filtrate heated with N/10 NaOH, 30 min...........	<5	32	86
Filtrate heated with N/10 NaOH, 60 min...........	7	23	51
Filtrate heated with N/10 HCl, 30 min.............	18	25	72
Filtrate heated with N/10 HCl, 60 min.............	7	30	90
Control...	<5	25	84

or alkali.   One drop of a heavy suspension of cells was added to each differently treated filtrate, so that an inoculum of one loop taken from it carried approximately 200,000 cells.   Results of this experiment showed that destruction of the growth-stimulating properties of the filtrate had occurred in every case (table 5). This is in marked contrast to the heat stability of the stimulative ingredients of yeast or azotobacter extract.

Activity of growth factor synthesized by R. trifolii

A quantitative study of the activity of *Rhizobium* culture filtrates in stimulating growth of the same organism was under-

taken, using a modified method for growth measurement. In this and subsequent experiments, twelve-ounce bottle plates were sown with giant colonies (see Plate 3). Usually, 11 or 12 colonies were sown to insure obtaining growth of at least 10. After 72 hours' incubation at 28°C., growth from ten colonies was removed and suspended in 10 ml. distilled water, and the turbidity read in an Evelyn electrophotometer. From a standardization curve the electrophotometric readings could be converted directly into numbers of cells. Parallel experiments were run, using fluid cultures as before, with the exception that growth was determined in the electrophotometer after 48 hours, instead of by direct count.

It was found more convenient in these studies to employ autolyzed cultures of the organism (8 to 10 weeks old) as a source of the growth factor. After the cells had settled, the supernatant medium was removed and, by pasteurization at 80°C. for 5 minutes, was rendered sterile without any destruction of stimulative activity. These culture autolysates were prepared from cultures grown in purely synthetic media for over ten transfers.

To determine the potency of the culture autolysate as a stimulant for *R. trifolii* it was tested at concentrations of .0001, .001, .01, .1 and 1.0 ml. per 10 ml. of medium. Additions were made both before and after autoclaving, to compare the effects of heated and unheated autolysate at various concentrations. At the same time, an autolysate of *Azotobacter chroococcum* prepared in the same manner, was tested to determine whether or not this organism also synthesized the factor.

The results (fig. 4) indicated that the *Rhizobium* autolysate was most active when added to the medium in concentrations between .001 and .01 ml. per 10 ml. of medium. The optimum concentration of autolysate (which is approximately equal to the amount of medium carried from an old to a new culture in a loop inoculation) is the same whether determined by plate or fluid cultures. Of interest, is the observation that additions beyond 0.1 per cent resulted in loss of stimulative action; this is

a peculiarity which assisted in identifying the active material. Autoclaving inactivated the autolysate at all concentrations.

Azotobacter autolysate apparently contains very little, if any, of the heat-labile *Rhizobium* factor, but does cause response at higher concentrations due to the presence of a heat stable substance, presumably that described by Allison and Hoover.

Although *Azobacter* did not synthesize appreciable quantities of the *Rhizobium* factor, it was thought possible that a more
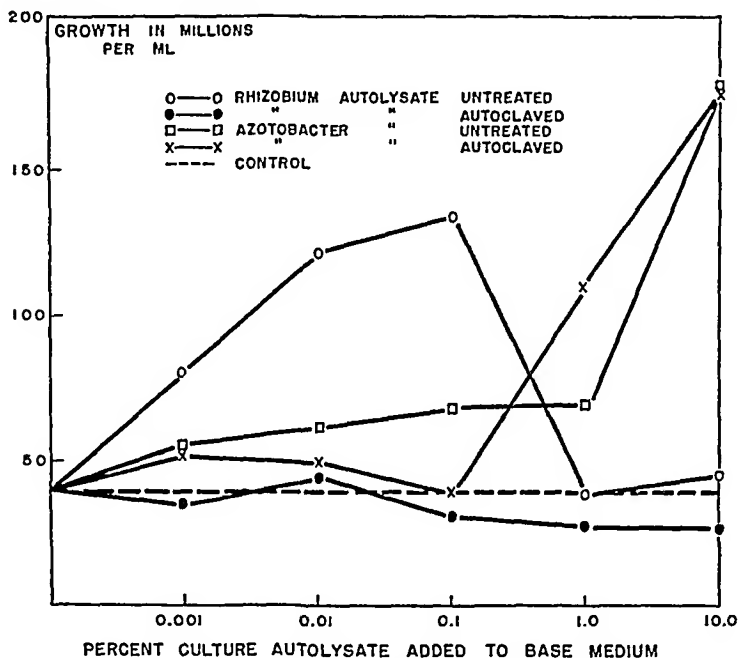
FIG. 4. Effect of heat on growth stimulants synthesized by *Rhizobium trifolii* and *Azotobacter chroococcum* determined by the giant colony method.

closely related species such as *Phytomonas tumefaciens* might do so, unless the phenomenon were strictly confined to the nodule organisms. The effects of *Phytomonas* autolysate on the growth of *R. trifolii* as determined by the giant colony technic are shown in figure 5. It appears that *Phytomonas tumefaciens* synthesizes the stimulative material in approximately the same quantity as do the rhizobia themselves.

Thiamin and riboflavin as growth factors for R. trifolii

From certain of the properties already discussed, it appeared possible that the growth activator synthesized by the rhizobia might be some known constituent of the vitamin B group, such as thiamin or riboflavin (or the unstable complexes which these substances form in the living cell). In order to test this hypothesis, inocula for both giant colony and fluid cultures were employed consisting of washed cells, which permitted detection of the factor or factors which could replace the material syn-
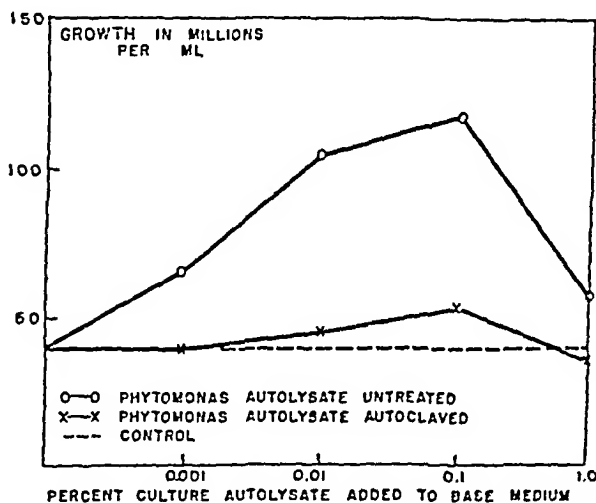
FIG. 5. Synthesis of *Rhizobium* factor by *Phytomonas tumefaciens*

thesized by the organisms. Pure thiamin and riboflavin were used in these experiments.

In preliminary studies, both of these substances were found to possess marked activity, but the organisms exhibited great sensitivity to only slight alterations in the concentration of the activator. The activity of these vitamins appeared to be limited to a narrow range of concentrations around 0.1 microgram per ml. This range of activity was studied more closely with the results, for thiamin, shown in Table 6. A similar experiment with both thiamin and flavin is shown in figure 6. In all cases it will be observed that as the concentration of either vitamin

increases beyond the optimum its stimulatory effect decreases.
This is of interest in view of the fact that the factor or factors
synthesized by the rhizobia possess the same peculiarity.   This
effect is shown clearly in the accompanying Plates 2 and 3.

If thiamin and flavin are the actual substances synthesized by
R. trifolii, it would be expected that they would not give further
stimulation of growth if the inoculum were taken direct from a
growing culture.   This was found to be the case (see table 6).
In the presence of sufficient of the activator, further additions

TABLE 6

Influence of inoculum on response of Rhizobium trifolii to vitamin B_1 (thiamin)
Counts (millions per ml.) on 48-hour fluid cultures calculated from
electrophotometer turbidity measurements

MEDIA, VITAMIN B_1	INOCULUM	
	Washed cells	Growing culture
micrograms / ml.		
0.2	65	161
0.18	81	149
0.16	140	114
0.14	141	127
0.12	150	123
0.10	155	110
0.08	147	116
0.06	106	116
0.04	64	155
0.02	50	147
0.00 (control)	43	145

had either very little stimulative influence, or actually depressed
growth.   The fact that vitamin B_1 has very little effect when
inocula are from actively growing fluid cultures has already been
reported by Laird and West (1938).

From these and other experiments which gave similar results,
it was concluded that either thiamin or flavin alone can replace,
at least in part, the culture autolysate, thus enabling washed
cell suspensions to grow nearly as readily in the reduced medium
as cells inoculated directly from an actively growing culture.

Synthesis of vitamin B₁ and flavin by R. trifolii

Since thiamin and flavin were strongly suspected of being identical with the *Rhizobium* factor, it became necessary to determine the ability of the organism to synthesize these vitamins. For this purpose, a very sensitive quantitative method for assaying vitamin B₁ was developed, based on Knight's finding (1937) that in appropriate media, the growth of *Staphylococcus aureus* is proportional to the amount of vitamin B₁ present.
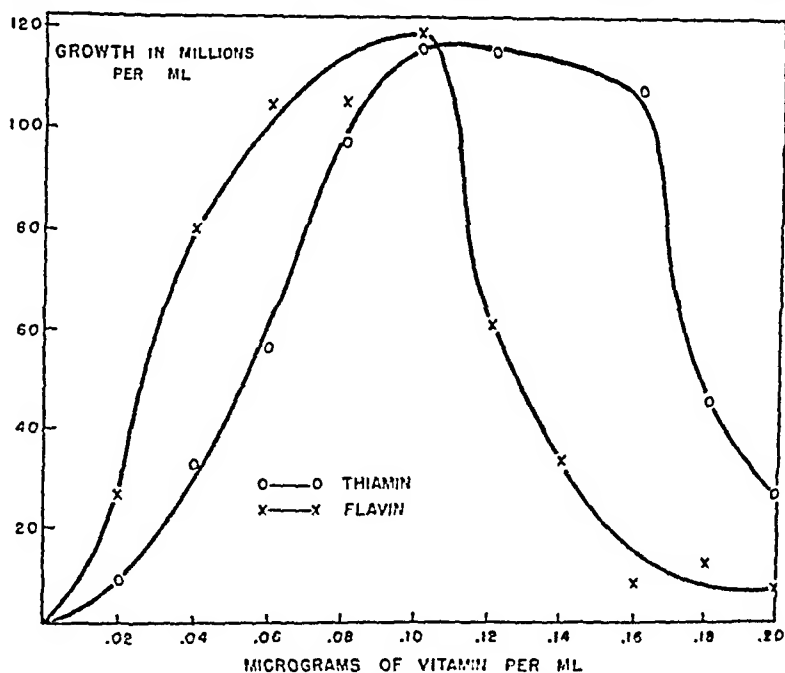
FIG. 6. Influence of pure thiamin and flavin on growth of *Rhizobium trifolii*

Details of this procedure are reported elsewhere (West and Wilson, 1938). Results of analyses of *R. trifolii* cultures grown on a synthetic, vitamin-free medium indicated an average of 19.6 micrograms of vitamin B₁ per gram of dry cells. This relatively high value indicates that the vitamin B₁ content of *R. trifolii* cells closely approximate that of yeasts.

The flavin content of *R. trifolii* was estimated by the method developed by Snell (unpublished data) involving growth re-

sponse and acid production by *Lactobacillus casei*.   An average from four separate determinations showed the flavin content of *Rhizobium* cells to be 0.370 microgram per milligram dry cells. These cells are therefore rich in flavin, since the *Clostridium butyricum*, reported by Warburg and Christian (1933) to be high in flavin, contained only .09 microgram per milligram.

Since *R. trifolii* synthesizes thiamin and flavin in such appreciable amounts, it would appear that these substances must be of great importance in the metabolism of the organism.   Possibly, one of the reasons for the existence of the lag phase in *Rhizobium* cultures is the necessity for synthesis of sufficient of these stimulants before multiplication can occur.   Therefore, if the inoculum be washed, and the cells deprived of most of these growth factors which would ordinarily be carried from the previous culture, the lag is prolonged, in many cases indefinitely.   The addition of vitamin B_1, flavin or culture filtrate overcomes this inactivity.

Nature of the growth factor synthesized by Rhizobium trifolii

From a comparison of the thiamin and flavin contents of the autolysates from *Rhizobium trifolii* cultures and the amounts of these vitamins which must be added in pure form to produce stimulation, it was found that more of either vitamin is required for growth response when added singly than occurs in the bacterial autolysates.   There are two possibilities to account for this apparent discrepancy: (*a*) thiamin and flavin in combination may have a greater than additive effect; (*b*) these vitamins may exist as protein or other complexes with greater activity than they possess in the free state.   Evidence has been obtained that both of these possibilities may be involved.

Experiments on the combined effects of thiamin and flavin showed that if either vitamin is present in optimum concentration, addition of the other reduces activity.   However, if both are present in amounts too small to be effective alone, the combination is stimulative.   This interaction of flavin and thiamin is shown in figure 7.

It would seem that thiamin is present in the cell as an unstable

complex since, in assaying autolysates from *Rhizobium* culture
for vitamin B₁, it was necessary to heat for 5 minutes (N·10
hydrochloric acid) at 100°C., a treatment liberating the free
vitamin but not destroying it.   On assaying the unheated au-
tolysate, only 0.3 microgram of vitamin B₁, per gram of dry cells
was found, whereas after a short heating the vitamin B₁, was
freed (19.6 micrograms per gram).²   In assaying for flavin it
was likewise necessary first to liberate the free substance by heat
treatment.

In combined form the vitamins are apparently more stimula-
tive since, following the splitting of these complexes by heat, a
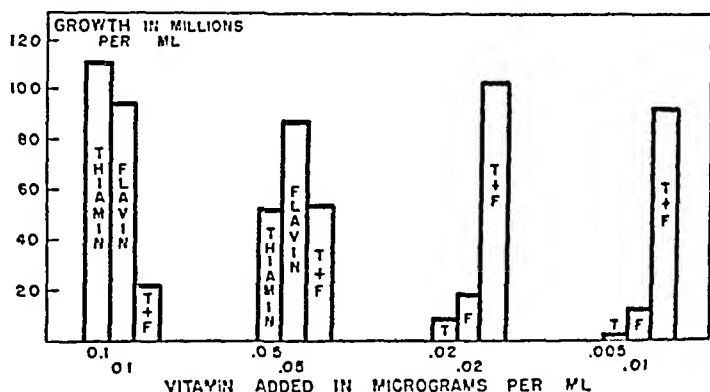
FIG. 7. Effect of combinations of thiamin and flavin on *Rhizobium trifolii* as
determined by growth in fluid culture.

treatment which is sufficient to liberate the free vitamin but
insufficient to destroy it, the activity of the *Rhizobium* autolysate
is largely lost.

For these reasons it can be well understood that either flavin
or thiamin alone does not possess the same degree of activity as
the culture autolysate which may contain both of these vitamins
in correct combination and in more suitable form.

² Recent work by Sinclair (1938) indicates that the vitamin B₁ of blood is also
in combined form (not co-carboxylase).   In assaying B₁ of blood by Meiklejohn's
method it is necessary to subject it to a short heating capable of liberating the
vitamin but insufficient to cause any destruction of the vitamin itself.

DISCUSSION

The more important results of this investigation may be summarized as follows:

1. Heat- and alkali-labile substances are synthesized by *R. trifolii* itself, which, when present in sufficient amount in the inoculum, permit considerable growth of the organism in a medium of purified ingredients. Even small inocula (200,000 to 500,000 per 10 ml.), washed free of the metabolic products of the surrounding medium, initiate growth satisfactorily if they are suspended in a filtrate from a growing culture. In fluid culture, the total population in this case may reach as high as 250–400 million per ml. in 72 hours. If the culture filtrate is merely added to the synthetic medium, the total population reaches only 100–150 million organisms per ml. in 72 hours. These essential factors occurring in the filtrate from an actively growing culture on synthetic medium can be replaced, at least partially, by the direct addition of riboflavin and thiamin in suitable concentration to the synthetic medium. Washed cells in the presence of the vitamin-enriched medium reach a density of 75 to 150 millions per ml. in 3 days. In the absence of culture filtrate, thiamin, or riboflavin, little or no growth occurs with such an inoculum.

The factor essential in growth initiation of *R. trifolii*, which is synthesized by actively growing cultures of the same organism has been termed the *Rhizobium* factor in order to distinguish it from the heat-stable substance of Allison and Hoover. The *Rhizobium* factor presumably consists of thiamin and riboflavin and possibly some other unidentified compounds, all of which the organism is able to synthesize, *once growth has been initiated.*

The use of the term "essential" as applied to the *Rhizobium* factor may appear to be somewhat arbitrary. It is used in the sense that the factor (or factors) must be present in order that growth may be initiated in a properly poised synthetic medium. Ordinarily, sufficient is carried over in a loop inoculum directly from an actively growing culture, and in this case the essential factor is furnished by the organisms themselves. If the organism is separated from its previously synthesized growth factor, it must be added to the medium, else little or no growth occurs.

But for initiation of growth in the synthetic medium the "essential" factor must be present, whether supplied directly in preparation of the medium or indirectly in the inoculum. In either case, once growth is initiated, the organism can synthesize sufficient of the factor to enable it to be transferred continuously even though maximum populations are not reached.

2. A second factor described by Allison and Hoover is also concerned with the nutrition of *R. trifolii*. This factor, termed "Coenzyme R," stimulates the growth of these bacteria, but does not appear to be essential for their continuous transfer. In the presence of optimum quantities of the factor of Allison and Hoover, the total population may reach 300–750 millions of organisms per ml. in 3 days. "Coenzyme R" differs markedly from the *Rhizobium* factor, and the two can be readily distinguished on the basis of stability and physiological effects.

It is possible that the heat-stable and heat-labile factors may be related as, for example, the factor described by Allison and Hoover may provide an organic nucleus which the organism converts readily into its necessary growth factors. According to Allison and Hoover "Coenzyme R" is concerned with respiration; the established importance of both thiamin and riboflavin to respiratory processes might be used as supporting this suggestion of relationship. However, much more experimentation will be required to investigate this phase of the subject.

3. In the light of these studies on the importance of condition of inoculum in determining whether or not growth will occur in a synthetic medium, it is believed that many of the discrepancies occurring in the literature on this point can be explained. For example, Thorne and Walker were able to obtain continuous transfer in synthetic medium starting with an inoculum from a yeast-extract slant and subsequently transferring one ml. of the 4 day old culture to 25 ml. of fresh liquid medium. Objection to their work on the grounds that they were transferring "Coenzyme R" from the original yeast extract culture can hardly be sustained, since they carried the organisms through 10 to 20 transfers. Under even more rigorous conditions with respect to size of inoculum, we have maintained continuous culture for over 30 transfers with no evidence of loss in reproductive power.

Nilsson, *et al.* (1938a), on the other hand, have recently reported complete inability to obtain growth on a synthetic medium even in the presence of reducing substances; they attribute the success of other workers to faulty technique.   Although they do not give much detail regarding the preparation of their inoculum, they do state that they used few cells, obtained by dilution of a culture.   This probably corresponds to our inoculum of cells suspended in fresh medium and therefore deficient in *Rhizobium* factor.   We are in complete agreement with them that under such conditions no growth results even on inoculation into an adequately reduced medium.   And it is also understandable that under such conditions they find vitamin B$_1$ stimulative (Nilsson, Bjälfve and Burström (1938b), since it is only when an inoculum deficient in the *Rhizobium* factor is employed, that vitamin B$_1$ induces any marked response.

SUMMARY

1. *Rhizobium trifolii* synthesizes all the organic substances essential for its growth from the simple ingredients of a properly reduced carbohydrate mineral-salts medium.

2. Although various tissue and microbial extracts contain a heat-stable substance (or substances) stimulative to growth, these are not required for successful continued transfer of the organism in a synthetic medium.

3. Continuous transfer of *Rhizobium trifolii* in a synthetic medium is dependent on a factor synthesized by the growing culture which is ordinarily transferred in sufficient amount in the inoculum to stimulate growth initiation.

4. This factor can be separated from cultures of *Rhizobium* by ultrafiltration.   It is readily destroyed by heat.

5. Small inocula, washed free of metabolic products of the culture from which they were taken, produce little or no growth in the absence of the *Rhizobium* factor.

6. In the presence of certain specific amounts of thiamin or flavin, either vitamin alone is capable of replacing to some extent the stimulative material synthesized by *R. trifolii*.   However, certain combinations of both vitamins are more active than either one used singly.

7. *Rhizobium trifolii* synthesizes appreciable amounts of both vitamin B_1 and riboflavin. These vitamins are present in culture autolysates or filtrates as complexes which are readily destroyed by heat, resulting in decreased activity.

8. Further evidence is presented which indicates that the activity of the metabolic products of *R. trifolii*, in stimulating growth initiation of the same organism, is due, at least in part, to the presence of thiamin and flavin in those products.

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PLATE 1

Influence of various supplements (25 parts per million) on growth of giant colonies of *Rhizobium trifolii*. A: Control. B: Casein hydrolysate. C: Thioglycollic acid. D and E: Allison's Azotobacter extract. F: Yeast extract.
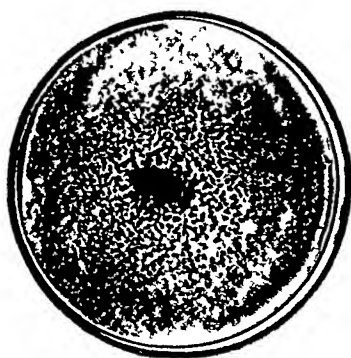
(P. M West and P W Wilson Growth factor requirements of bacteria)

PLATE 2

Stimulation of growth of *Rhizobium trifolii* in base medium around blocks containing 0.2 microgram per ml. vitamin B_1 and flavin.
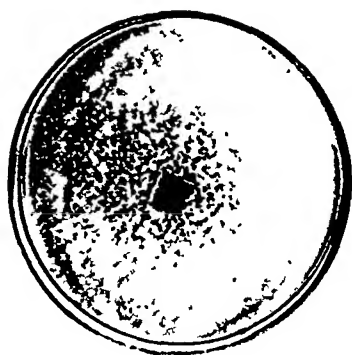
VITAMIN B₁   FLAVIN
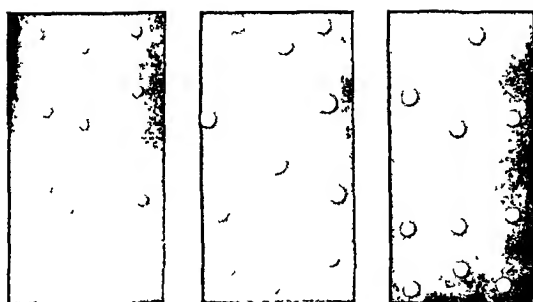
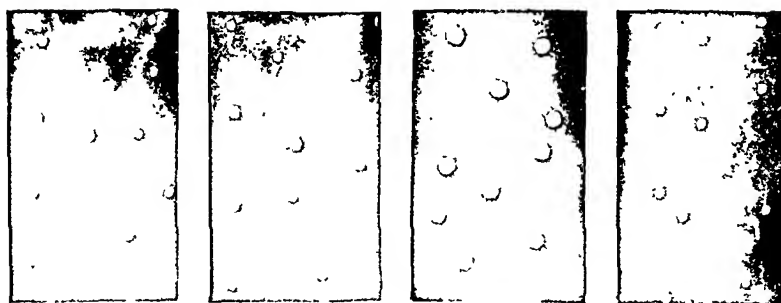(P. M. West and P. W. Wilson: Growth factor requirements of bacteria)

PLATE 3

Effect of varying concentration of vitamins on growth of *Rhizobium trifolii*. 1 and 4: Controls.   2 and 3: 0.1 and 0.2 microgram vitamin B_1 per ml.   5, 6, and 7: 0.05. 0.1 and 0.5 microgram flavin per ml.

(P. M. West and P. W. Wilson: Growth factor requirements of bacteria)

THE DETERMINATION OF HISTAMINE IN BACTERIAL CULTURES

ARNOLD H. EGGERTH, RALPH J. LITTWIN, AND JOYCE V. DEUTSCH

Department of Bacteriology, Long Island College of Medicine, Brooklyn, New York

The production of histamine in bacterial cultures has been the subject of a number of investigations. According to these reports, the power to produce this amine is confined to certain strains of only a few species of bacteria: *Escherichia coli* and related organisms (Mellanby and Twort, 1912–13; Koessler and Hanke, 1919c; Hirai, 1933); *Salmonella enteriditis, S. schottmuelleri*, and *S. morgani* (Koessler, Hanke, and Sheppard, 1928); a variety of *Aerobacter aerogenes* designated as *B. aminophilus-intestinalis* (Bertrand and Berthelot, 1913; Jones, 1918); and *Clostridium welchii* (Kendall and Schmitt, 1926; Kendall and Gebauer, 1930).

Several methods have been used to identify histamine in cultures. Ackermann (1910), Bertrand and Berthelot (1913), Mellanby and Twort (1912–13), Hirai (1933), and Kendall and Gebauer (1930), prepared the crystalline di-picrate of the base. This method, while reliable when positive, is qualitative only, because of the large losses in purification. The process is laborious, and rather large quantities of culture must be worked up.

Mellanby and Twort (1912–13) and Jones (1918) also determined histamine in cultures by the Dale and Laidlaw (1910–11) technic, employing the isolated guinea pig uterus. Kendall and Schmitt (1926) used guinea pig intestine in a similar technic. These, as well as other physiological methods, are open to the objection that substances other than histamine may provoke the same response. As Best and McHenry (1931) point out, no one single physiological method for histamine assay is reliable; if the

physiological method is to be used, only a complete biological analysis can establish the presence of histamine.

Koessler and Hanke (1919a) determined histamine colorimetrically by the Pauly diazo reaction. As many other substances react with the diazo reagent, it was first necessary for them to isolate the histamine in a fairly pure form (Koessler and Hanke, 1919b). This was done by extracting the cultures with amyl alcohol after making them strongly alkaline. The histamine was recovered from the amyl alcohol by shaking with dilute acid. It was then determined quantitatively by means of the diazo reaction, for which they described an improved technic.

In our investigation of histamine production by bacteria, we first made use of the extraction method of Koessler and Hanke (1919b). It was soon found that this method presented certain difficulties. It was very time-consuming. It required large amounts of reagents, some of which are expensive. The colors obtained with the extracts often do not match well with the standard, especially if glucose is present in the culture medium. We therefore changed the extraction method to overcome these drawbacks.

As extracting solvent, we used a mixture of three parts of chloroform to one part of amyl alcohol. "Reagent" grades of these solvents were used; after being mixed, they were shaken with about one-fifth of their volume of 1 per cent sulfuric acid; then with several changes of distilled water. This removes impurities that would be extracted later with the histamine.

This mixture of chloroform and amyl alcohol is a more selective solvent for histamine than is amyl alcohol alone. Its use avoids the extraction of the interfering substance produced from glucose, as well as some other interfering substances.

For the extraction, we used the excellent method devised by Widmark (1926), by which quantitative extractions of small amounts of material can be made with a minimum of labor and attention. Widmark's apparatus consists of two small connected chambers; in one of these is the solution to be extracted; in the other is placed a solution in which the extracted substance is

changed to a form insoluble in the extracting solvent.   A suitable immiscible organic solvent connects the two chambers; when filled, the apparatus is tilted continually, so that the extracting solvent flows back and forth between the two solutions.   The apparatus described by Widmark was intended for solvents lighter than water; as the chloroform-amyl alcohol mixture is heavier than water, we devised a different form of apparatus. This consists of two glass bulbs connected by a vertical U-tube
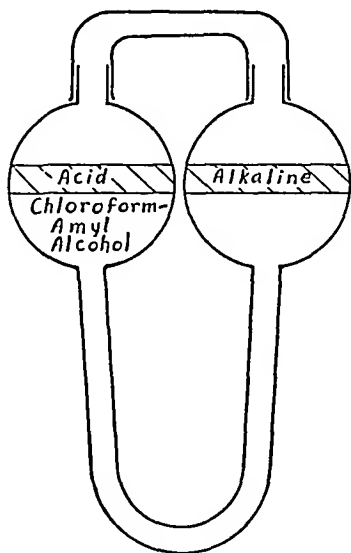

FIG. 1. DOUBLE BULB EXTRACTION APPARATUS

(fig. 1).   The bulbs have a diameter of 4.5 to 5.0 cm., a size that is suitable for extracting 10 cc. of fluid.   The removable tube connecting the two orifices was added to prevent loss of chloroform by evaporation.

The tilting apparatus consists of a rack with slots to hold 12 such "double bulbs."   The rack is pivoted in the middle; the tilting is done by a motor with a reducing gear, which tilts the rack 8 times per minute through an angle of 8 degrees on each side of the horizontal.

By our method, four main steps are involved in the determination of histamine in bacterial cultures:

I. The preparation of the acid extract.
II. The removal of volatile bases and amyl alcohol.
III. The quantitative determination of histamine by means of the diazo reaction.
IV. The preparation of histamine di-picrate from the acid extract (not done in every case.)

I. THE PREPARATION OF THE ACID EXTRACT

About 30 cc. of the washed chloroform-amyl alcohol mixture were introduced into a double bulb. Then, 10 cc. of 0.5 per cent sulfuric acid were pipetted into one bulb. Ten cubic centimeters of the test fluid (bacterial culture) were neutralized, then made alkaline by adding 1.5 cc. of 15 per cent Na_2CO_3 solution, and introduced into the other bulb. (Stronger alkali must not be used, otherwise interfering substances are extracted and the histamine readings become too low.)

The connecting bridge over the two orifices was then put into place, and the double bulb set in a slot in the tilting rack. The apparatus was then run for 24 hours at room temperature.

As the solution to be extracted is strongly alkaline, the histamine is present as the free base. This is slightly soluble in the extracting fluid; and a little of it dissolves in the chloroform-amyl alcohol at the interface. The tilting moves the extracted histamine to the other bulb, where it comes in contact with the dilute sulfuric acid, forming the sulfate. This is no longer soluble in the extracting fluid; it enters the aqueous layer, from which it cannot return. This process continues until all of the histamine is in the acid extract. Other bases, such as ammonia and the volatile amines, will also be transported, but the great bulk of impurities will be left behind. Not the slightest trace of such substances as histidine, peptone, etc., ever appears in the acid extract. The presence of proteins in no wise interferes. The chloroform amyl-alcohol layer often becomes very turbid during the extraction; this turbidity has no significance and can be disregarded.

Sulfuric acid is the acid of choice in this extraction, as none of it will be transported to the alkaline side.   Organic acids that are soluble in the choloroform amyl-alcohol will be rapidly carried over.   Hydrochloric acid is transported slowly; it can be used in this extraction if allowance is made for the fact that some of it will pass over to the alkaline bulb.

Factors that influence the speed of extraction:

The first factor that influences the speed with which histamine will be extracted is the composition of the extracting fluid.   Pure chloroform, in this apparatus, will extract histamine, but the rate is too slow to be of use.   The addition of as little as 5 per cent of amyl alcohol greatly increases the speed of extraction.   Our experience indicates that 25 per cent of amyl alcohol is most practical.   With still more amyl alcohol, the speed of extraction is increased, but the specific gravity of the mixture becomes dangerously low; more interfering impurities will also be extracted.

Either the normal or the iso-amyl alcohol can be used; we found no difference.   Butyl alcohol may be substituted for the amyl alcohol.   Even ethyl or methyl alcohol can be utilized; with these lower alcohols, much of the alcohol will be in the aqueous layers. Instead of chloroform, one may use tetra-chlor-ethane or carbon tetrachloride.   In our hands, however, chloroform and amyl alcohol gave the most satisfactory results.

Another important factor that determines the speed of extraction is the ratio of the area of the extracting surface to the volume of fluid to be extracted.   This ratio should be high; in other words, the fluid to be extracted must form a *shallow* layer. In our apparatus, this layer was less than 1 cm. deep.

Other factors that influence the rate of extraction are the speed of tilting, the angle of tilt, and the internal diameter of the connecting U-tube.   A large quantity of the extracting solvent must stream from one bulb to the other at each tilt of the rack. If the rate of tilting is too rapid, or if the angle of tilt is insufficient, or if the connecting U-tube is too narrow, then only small quantities of solvent will surge back and forth and the

rate of extraction is diminished. With our apparatus, 8 to 15 tilts per minute were found optimal.

We determined the rate of extraction of histamine in two different ways. By the first method, pure solutions of histamine di-hydrochloride were extracted for different periods and both the acid extract and the alkaline residue were assayed. By

TABLE 1

Rate of extraction of histamine from a solution containing 0.730 mgm. of histamine di-hydrochloride in 10 cc.

	FOUND IN ACID EXTRACT		FOUND IN ALKALINE RESIDUE	IN CHLORO-FORM-AMYL ALCOHOL (BY DIFFERENCE)
	mgm.	per cent	mgm.	mgm.
2-hour extraction....................	0.370	50.7	0.240	0.120
4-hour extraction....................	0.490	67.1	0.150	0.090
8-hour extraction....................	0.690	94.5	0.020	0.020
18-hour extraction....................	0.730	100.0	0	0

TABLE 2

Rate of extraction of histamine from a culture of Salmonella schottmuelleri on meat extract-peptone-histidine-glucose medium

	FOUND IN ACID EXTRACT (10 cc.)		REMAINING IN ALKALINE RESIDUE* (10 cc.)
	mgm.	per cent	mgm.
2-hour extraction......................	0.640	28.8	1.580
4-hour extraction......................	1.050	47.7	1.150
8-hour extraction......................	1.610	73.9	0.570
12-hour extraction......................	1.870	83.9	0.360
24-hour extraction......................	2.13	98.1	0.040

* Determined by re-extracting the alkaline residue for 36 hours.

the second method, a culture of a histamine-producing bacterium was extracted for different time intervals; the acid extracts were removed and the alkaline residues were extracted again for 36 hours. The results of such experiments are shown in tables 1 and 2.

Table 1 shows that histamine is very rapidly extracted from solutions of pure histamine; in 8 hours, 94.5 per cent was removed,

and within 18 hours, all of it. Extraction of cultures, however, takes place more slowly. In 8 hours, only 73.9 per cent of what was probably the total histamine was extracted; even after 24 hours, a slight trace was left. According to table 2, 0.040 mgm. of histamine was recovered by a 36-hour extraction following a 24-hour extraction (this figure may be too high, as the color match with the standard was only fair, and some part of this color value was undoubtedly due to some substance not histamine).

When a known amount of histamine is added to a bacterial culture, the added histamine is removed quantitatively by a 24-hour extraction.

On the basis of these and other similar experiments, we have concluded that this extraction method will remove all but traces of histamine from bacterial cultures in 24 hours.

II. THE REMOVAL OF VOLATILE BASES AND AMYL ALCOHOL

As stated before, the acid extract from bacterial cultures will contain considerable amounts of ammonia and volatile amines, which react with the diazo reagent. They can be readily removed by boiling at a slightly alkaline reaction. This process at the same time removes amyl alcohol, which also interferes. Our procedure was as follows:

The acid extract was removed with a capillary pipette and the bulb rinsed with a few cubic centimeters of distilled water. To this, we added a determined amount of 2 per cent Na_2CO_3 solution, that would, after boiling, exactly neutralize to phenolphthalein the 10 cc. of 0.5 per cent sulfuric acid used in the extraction. Finally we added 0.5 cc. of 2 per cent borax solution. (This amount of borax does not interfere with the diazo reaction, though a larger amount will do so.) The fluid was then transferred to a special boiling tube (fig. 2) in which it was boiled vigorously until volatile bases no longer appeared in the vapors. To determine this point, we used as indicator a solution of Brom-Cresol-Green, which was adjusted with minimal acid until its color was a yellowish green. A platinum loopful of this indicator, held in the escaping vapors, will turn blue as long as

volatile bases are coming off. Five to 8 minutes of vigorous boiling usually suffices. The pH of the fluid should be near 9.2. The volume was then made up to 10 cc.; the fluid was now ready for the colorimetric determination.

The boiling tube shown in figure 2 was devised to prevent bumping, which is otherwise very troublesome. It is based upon the principle of creating a "hot spot" and preventing general superheating of the entire bottom. This principle has been
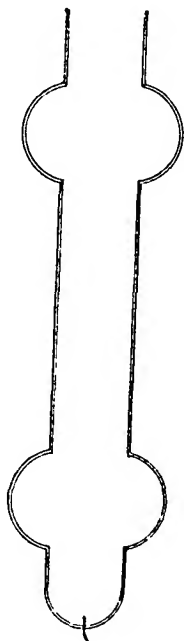

FIG. 2. BOILING TUBE WITH PLATINUM WIRE THROUGH BOTTOM

described by Moroney (1934) in connection with a quite different form of apparatus. The boiling tube was made from a thick walled Pyrex test tube, 200 by 25 mm. in size; a pin hole was blown through the bottom and a short piece of platinum wire passed through it. The hot glass was pinched down on the wire with heavy forceps, and the bottom worked in the flame until a smooth closure was obtained. The wire within the tube must be bare of glass. We have had no trouble with breakage

or leakage of these tubes. A small flame, passing through an 8 mm. hole in an asbestos gauze, is directed on the wire. Fluid can be boiled violently in this tube without any bumping. As some of our extracts frothed a good deal on boiling, the two expansions shown in the figure were blown in the tube.

Histamine solutions can be boiled for a long time in slightly alkaline reactions (pH 9.0 to 10.0) without loss of the amine. With greater alkalinity (pH 10.5 to 11.0 or more) some histamine is destroyed. We found that the removal of volatile bases by the evaporation of a neutral or alkaline solution was unsatisfactory, as there was always considerable loss of histamine. Only acid solutions of histamine can be evaporated to dryness without some loss.

III. THE COLORIMETRIC DETERMINATION OF HISTAMINE

We have followed exactly the method of Koessler and Hanke (1919a) for the diazo reaction and the colorimetric determination of histamine, which we have found very satisfactory. Our chief difficulty, at first, was that small quantities of histamine gave color values that were too low; after re-crystallizing the sulfanilic acid twice from hot water, this difficulty disappeared. For complete details, we refer the reader to Koessler and Hanke's article (1919a). Briefly, their technic is as follows:

Reagents for the test:
   0.9 per cent sulfanilic acid in 10 per cent HCl.
   5.0 per cent $NaNO_2$ solution.
   1.1 per cent Na_2CO_3 solution.
The diazo reagent is prepared by measuring 1.5 cc. of the sulfanilic acid solution into a flask in an ice bath; then 1.5 cc. of the 5 per cent $NaNO_2$ solution are added. After 5 minutes, another 6.0 cc. of the $NaNO_2$ solution are added. In 5 more minutes, the reagent is made up to 50 cc. with cold distilled water. It is ready for use in 15 minutes.
   The diazo reaction:
Five cubic centimeters of the 1.1 per cent Na_2CO_3 solution and $(1 - x)$ cubic centimeters of water are measured into a cylinder of the colorimeter (x is the volume of extract to be tested). Two cubic centimeters of the diazo reagent are added, within the space of 5 seconds, to the

alkali in the cylinder and mixed, noting the time to the second. (A stopwatch should be used.)   In exactly 1 minute, x cc. of the test fluid are added and mixed.   With histamine, the color is first yellow, then red; it is at its maximum in 4 to 5 minutes, after which it fades.   The reading is made at the end of 5 minutes after the addition of the test fluid.

The volume x of test fluid should be chosen so that, if possible, the colorimetric reading falls between 5 and 20 mm.   This can be determined by a rapid preliminary test: in quick succession, add 1 drop of the sulfanilic acid solution, 5 drops of the $NaNO_2$ solution, and 5 cc. of the Na_2CO_3 solution; then the test fluid is added drop by drop until the desired depth of color is obtained.

For standard solution we used the Congo-Red Methyl-Orange standard of Koessler and Hanke (1919a), which we also found very satisfactory.   Two solutions are prepared: a Congo Red solution (2.500 grams of Grubler's Congo Red dissolved in 50 cc. of absolute alcohol, then water added to 500 cc.), and a Methyl Orange solution (0.500 grams of Methyl Orange in 500 cc. of water).   The standard is made by adding 1.0 cc. of the Congo Red solution and 1.1 cc. of the Methyl Orange solution to about 250 cc. of water and diluting to 500 cc.   This color standard deteriorates slowly and should be checked daily.   For this purpose, a 1:10,000 solution of histamine di-hydrochloride, layered with toluene, is kept on hand; 0.25 cc. of this should give a reading of 18.7 mm.

To make the reading, the cup with the test solution is set at 20 mm.; the cup with the standard is moved until a match is found.   A correction of 0.3 mm. is subtracted from the reading of the standard to allow for the color of the reagent.   Tables given by Koessler and Hanke (1919a) enable one to calculate the concentration of histamine (as the di-hydrochloride) in the extract.   Or the concentration may be calculated by the following formula:

$$\frac{\text{Reading of standard cup in mm.}}{\text{Volume of fluid tested in cc.}} \times 1\tfrac{1}{3} = \text{gammas of histamine di-hydrochloride per cc. of test solution.}$$

IV. THE PREPARATION OF HISTAMINE DI-PICRATE
FROM THE ACID EXTRACT

As the identification of histamine is not complete unless a crystalline salt is prepared and identified by its melting point and mixed melting point with crystals of pure histamine salt, we prepared the histamine di-picrate from the acid extracts of cultures of all organisms that produced histamine in good yield. As the acid extracts are free from interfering substances, the di-picrates could be prepared and purified with ease.

It was our custom to grow each organism on several different kinds of media, and at several different temperatures. Consequently, when an organism was found positive for histamine, there were always ten or more tubes of extract available for preparing the di-picrate. After the colorimetric determinations were made, the remainders of such positive extracts were combined, adjusted to pH 6.0, and evaporated until the concentration of histamine was approximately 1 part in 1000. To every 9 cc. of this solution, 1 cc. of 10 per cent picric acid in methyl alcohol was added, and the mixture cooled over night. The di-picrates were re-crystallized from hot water 2 to 5 times until their melting points and mixed melting points were 239 to 241°C.

The dry histamine di-picrate can also be crystallized from absolute methyl alcohol. As the quantities were usually small, all crystallizations were done in small test tubes; the crystals were packed on the centrifuge and the supernatant fluids poured off. When the correct melting points were obtained, the di-picrates were dissolved in water and tested with the diazo reagent; in every case the characteristic red color was obtained.

DISCUSSION

When bacteria act upon histidine, it is quite possible that imidazols other than histamine may be produced. Inasmuch as these, if present in the acid extract, would produce a color like that of histamine, we felt it necessary to prepare and study such other derivatives of histidine, as follows:

Imidazol propionic acid. Found by Bertrand and Berthelot (1913) in cultures of their *Bacillus aminophilus*. It was prepared from imidazol lactic acid by the method of Knoop and Windaus (1905).

Imidazol lactic acid. Found in bacterial cultures by Hirai (1933). It was prepared by the method of Fränkel (1903).

Urocanic acid. Found in bacterial cultures by Raistrick (1917). It was prepared by his method from histidine by the action of *Salmonella paratyphi.*

Imidazol. Imidazol and methyl imidazol have not been described as occurring in bacterial cultures; but as they are the analogs of indol and scatol, they are possible bacterial metabolites. Imidazol was prepared from glyoxal sulfate by the method of Ruggli and Henzi (1929); the oxalate was prepared and crystallized from dilute acetone and dilute alcohol; m.p. 234°C.

4-Methyl imidazol. This was prepared by the method of Bernhauer (1929).

(4-Ethyl imidazol and 4-vinyl imidazol are also possible metabolites of histidine. We did not prepare these two substances. They should resemble 4-methyl imidazol in their properties.)

Imidazol ethyl alcohol. According to Ehrlich (1911) this substance is formed by the action of yeast upon histidine. It was prepared by the method of Windaus and Opitz (1911).

Solutions of these 6 imidazols, as well as of histidine and histamine, were studied and compared in the following ways:

1. When made alkaline and extracted in our apparatus in the usual way, only histamine, imidazol, methyl imidazol, and imidazol ethyl alcohol appear in the acid extract. Not the slightest trace of histidine, imidazol propionic acid, imidazol lactic acid, or urocanic acid will appear in the acid extract.

2. When coupled with sulfanilic acid, histamine gives an orange red color that exactly matches the Congo-Red Methyl-Orange standard of Koessler and Hanke (1919a). The color with histidine, urocanic acid, and imidazol ethyl alcohol is only slightly more orange. Imidazol lactic acid, imidazol propionic acid, and methyl imidazol yield a redder color that matches the Congo Red alone. Imidazol, on the other hand, gives a deep orange color, suggesting that, with the 4-position of the nucleus not occupied by a side chain, the coupling may take place at a different point than with the other imidazols.

3. When 1:1000 solutions of these 8 imidazols were treated with phosphotungstic and sulfuric acids, immediate precipitates

appeared with all but the imidazol lactic acid; with the latter, a small precipitate appeared on cooling. When these precipitates were removed by centrifugation, and the supernatant fluids neutralized and tested with the diazo reagent, strong colors developed in every case except that of histamine. *Of these bases, only histamine is completely precipitated by phosphotungstic acid.*

When 1:5000 solutions of these imidazols were tested with phosphotungstic and sulfuric acids, an immediate precipitate appeared only with histamine and imidazol ethyl alcohol. On standing in the refrigerator, small precipitates formed with histidine, urocanic acid, imidazol, and methyl imidazol. In dilutions of 1:10,000, a precipitate formed with histamine only.

Histamine, as Koessler and Hanke (1920) have shown, is completely precipitated by phosphotungstic acid. None of the other 7 imidazols here studied are completely removed. The presence of any of them in an extract can, therefore, be readily demonstrated by testing the supernatant fluid after phosphotungstic acid precipitation (it is necessary only to neutralize the acid). Conversely, if the supernatant fluid does not give the characteristic color with the diazo reagent, these other imidazols are absent. *We made it a practice to test every fluid that gave a histamine-like color in this way.*

As will be explained in the followed paper, in no case did the phosphotungstic acid supernatants of the acid extract contain any imidazols when the culture medium contained an organic compound of nitrogen (such as asparagine or peptone) in addition to histidine itself. But in cultures on a medium in which ammonium salts and nitrates were the only other source of nitrogen, such imidazols were frequently found (though in small quantities) in the supernatant fluids following phosphotungstic acid precipitation.

4. With Nessler's reagent, no precipitate is formed with histidine, imidazol propionic acid, imidazol lactic acid, or urocanic acid in a concentration of 1:1000. White precipitates are formed with histamine, imidazol, methyl imidazol, and imidazol ethyl alcohol. Nessler's reagent will produce a precipitate in a

1:700,000 solution of histamine di-hydrochloride. A modified Nessler's solution, made up with 16 per cent of Na_2CO_3 instead of the usual 10 per cent NaOH, will still precipitate histamine, though it will not precipitate ammonia.

5. Imidazol, methyl imidazol, and imidazol ethyl alcohol are soluble in ether; histamine and the imidazol acids are not.

6. Only histamine and urocanic acid yield picrates that are highly insoluble in water.

In addition to these imidazols, we tested the behavior of certain other substances that are likely to be present in the acid extracts, as follows:

1. Koessler and Hanke (1919c) encountered a base derived from histidine to which they ascribed the formula $HCNH_2$: $CNH_2CH_2CH_2NH_2$, though they did not isolate it. We encountered what is probably the same base; it is found most abundantly in cultures of the Friedlander-aerogenes group of organisms; it is also formed by other bacteria when histidine is the only source of both nitrogen and carbon. This base appears in the acid extracts. With Nessler's reagent, it gives a heavy white precipitate which soon turns yellow. It is incompletely precipitated with phosphotungstic and sulfuric acids. The color formed with the diazo reagent is pale yellow. The presence of this base can usually be neglected, as the color formed by it is so pale.

2. Tyramine might be formed in certain media and appear in the acid extract. As dilute solutions of tyramine are not precipitated by phosphotungstic acid, a test on the supernatant fluid would reveal it. We have never found, with the media used by us, any indications of tyramine.

3. If phenols or cresols are present in the culture, a very small fraction of them will appear in the acid extract. They would not be precipitated by phosphotungstic acid. We have never found phenols or cresols in recognizable quantities in the acid extracts of our cultures (which contained glucose and hence were acid in reaction). Hanke and Koessler (1924) also found that phenols and cresols were not formed in media that became acid.

4. Certain media, especially those that contain peptone and meat extract, will yield an acid extract containing a small amount of color-producing substance. Usually this color is pale yellow or

pale orange. When it is small in amount, it will not interfere with the determination of histamine. It is only necessary then to make a correction of the final reading for the medium. Media that require a large correction (more than 5 to 6 mm. per cubic centimeter) should be avoided.

The method described above has been used in over two thousand determinations of histamine, the results of which are reported in a following paper. This method requires a minimum of attention, labor, and reagents. An extract is obtained which contains histamine in a high state of purity; consequently, the color matches with the standard are very satisfactory. Only where the amount of histamine is very low (less than 5 gamma per cubic centimeter of culture) are the color matches likely to be "fair" rather than "excellent." Histidine and other imidazol acids never appear in the acid extract. With one medium only were histamine-like colors obtained that were in part due to some imidazol base other than histamine; that one medium contained, in addition to histidine, only ammonium salts and nitrates as a source of nitrogen. In all other media where other organic compounds of nitrogen were supplied, the characteristic color was due to histamine alone.

SUMMARY

1. A method for the quantitative extraction of histamine from small quantities of bacterial cultures is described.

2. A quantitative determination of histamine is made on this extract by the colorimetric method of Koessler and Hanke. Histamine di-picrate can also be readily prepared from this extract for positive identification.

3. Various substances that might interfere with the determination of histamine have been studied, and the means of recognizing and eliminating them indicated.

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THE PRODUCTION OF HISTAMINE IN BACTERIAL CULTURES

ARNOLD H. EGGERTH

Department of Bacteriology, Long Island College of Medicine, Brooklyn, New York

In a previous paper (Eggerth, Littwin, and Deutsch, 1939) a simple and convenient method for the quantitative determination of histamine in bacterial cultures has been described. By this method, the cultures are made alkaline with sodium carbonate, then extracted in a special apparatus with a mixture of chloroform and amyl alcohol, the histamine passing into a layer of dilute sulfuric acid. Ammonia, volatile amines, and amyl alcohol are removed from this extract by boiling at pH 9.2. The histamine is determined colorimetrically by the method of Koessler and Hanke (1919a). The presence of histamine is then confirmed qualitatively by forming the di-picrate.

In the present investigation, a series of organisms has been studied to determine which of them are capable of producing histamine, and what factors influence histamine production.

A number of culture media were employed, as the composition of the medium profoundly affects the amount of histamine produced. A detailed report will be made of the following seven only. Histidine is an essential ingredient for all of them, for, with the exception of *Clostridium welchii*, none of the organisms studied were able to form histamine unless free histidine was present.[1]

The first five media were adjusted to pH 7.6 and autoclaved; then enough sterile concentrated glucose solution was added to

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[1] Suzuki and Joshimura (1909) have shown that infusions of the flesh of certain sea fish are rich in free histidine. In the present investigation, it was found that many organisms produced high yields of histamine on a medium consisting of fresh mackerel infusion with asparagine or peptone and glucose, with no added histidine.

give a concentration of 1 per cent. Medium 6 was also adjusted to pH 7.6, but 2 per cent of glycerol was added instead of the glucose. The media were then distributed in sterile tubes, 12 cc. per tube.

No. 1. Ammonium-nitrate-histidine-glucose medium:

> 300 cc. H_2O
> 0.3 gram histidine di-hydrochloride
> 0.3 gram $(NH_4)_2SO_4$
> 0.6 gram $NaNO_3$
> 0.6 gram $Na_2HPO_4 \cdot 12H_2O$
> 0.3 gram KCl
> 0.05 gram $CaCl_2$
> 0.05 gram $MgSO_4$

Many organisms failed to grow on this medium. A control determination made on the uninoculated medium gave no color with the diazo reagent.

No. 2. Asparagine-histidine-glucose medium:

> 300 cc. H_2O
> 1.0 gram asparagine
> 0.3 gram histidine di-hydrochloride
> 0.6 gram $Na_2HPO_4 \cdot 12H_2O$
> 0.3 gram KCl
> 0.05 gram $CaCl_2$
> 0.05 gram $MgSO_4$

A control determination on the uninoculated medium gave no color with the diazo reagent.

No. 3. Asparagine-histidine-cysteine-glucose medium. This has the same formula as medium 2, but with the addition of 0.3 gram cysteine hydrochloride.

No. 4. Egg-yolk-asparagine-histidine-glucose medium. Seven parts of distilled water were added to 1 part of fresh egg yolks and stirred. The mixture was heated in the Arnold sterilizer for 1 hour and filtered.

> 300 cc. egg yolk infusion
> 0.3 gram histidine di-hydrochloride
> 1.0 gram asparagine
> 0.6 gram $Na_2HPO_4 \cdot 12H_2O$
> 0.3 gram KCl
> 0.05 gram $MgSO_4$

A control determination on the uninoculated medium gave a color reading of 0.3 mm. per cubic centimeter. This medium gave excellent growth with most organisms, and the yields of histamine were high.

No. 5.   Meat-extract-peptone-histidine-glucose medium:

        300 cc. H₂O
        1.2 grams meat extract
        3.0 grams peptone (Parke Davis)
        0.3 gram histidine di-hydrochloride

A blank determination on the uninoculated medium gave a color reading of 5.6 mm. per cubic centimeter which matched the histamine standard fairly well.

No. 6.   Meat-extract-peptone-histidine-glycerol medium. This medium has the same formula as the preceding one, except that the carbohydrate added is 2 per cent glycerol.

No. 7.   Histidine alone, with no other source of nitrogen and no carbohydrate:

        300 cc. H₂O
        0.3 gram histidine di-hydrochloride
        1.2 grams Na₂HPO₄·12H₂O
        0.3 gram KCl
        0.05 gram CaCl₂
        0.05 gram MgSO₄

Acetic acid or sodium hydroxide were added to obtain the desired pH. Little or no growth is to be expected in this medium; it was used to determine histamine production by "resting" bacteria. A blank test on the uninoculated medium gave no color with the diazo reagent.

In calculating the amount of histamine produced in a culture, a correction was made in each case for the blank for that medium, in addition to a correction of 0.3 mm. for the color of the reagent.

I.   THE RATE OF HISTAMINE PRODUCTION IN BACTERIAL CULTURES

Koessler and Hanke (1919b) determined the rate of histamine production by *Escherichia coli* in a medium containing only

inorganic salts and glycerol in addition to histidine. In their experiment, histamine formation took place very slowly; none was formed in 2 days; in 5 days, only 3.3 per cent of the available histidine had been converted to histamine; in 10 days, 24.4 per cent; and in 40 days, 83.5 per cent.

When a richer medium is used, histamine production is much more rapid, as is shown in table 1.

Similar experiments with other organisms and other media show that on the richer media considerable histamine is formed

TABLE 1

Rate of the production of histamine by Shigella dysenteriae St. on medium 5 (meat extract-peptone-histidine-glucose) at 31°C.

	HISTAMINE PRO-DUCED (AS THE DI-HYDROCHLORIDE)	HISTIDINE CON-VERTED TO HISTAMINE
	mgm. per cc.	per cent
1-day culture	0.094	11.6
2-day culture	0.191	23.6
5-day culture	0.417	51.6
11-day culture	0.444	55.0
15-day culture	0.463	57.3
23-day culture	0.460	56.9
30-day culture	0.458	56.7

during the first 24 hours; that most of the production takes place within 5 days, and that the maximum yield is obtained in about 2 weeks.

II. THE EFFECT OF THE pH ON THE PRODUCTION OF HISTAMINE

The effect of the pH was tested in a variety of ways. The most satisfactory procedure was as follows: A small amount of glucose (0.3 per cent) was added to the medium. Eight to 10 hours after inoculation, when active growth had started, the cultures were adjusted to the desired pH by the addition of sterile 10 per cent acetic acid or 4 per cent sodium hydroxide. During the first 48 hours, the pH of a culture changes rapidly, and the pH must be adjusted every 6 to 8 hours; after that, two or even one daily adjustment suffices. To determine the pH,

large loopfuls of the cultures were mixed with drops of indicator on a spot plate; the colors were then compared with those produced in a similar way by known buffer solutions.

The results thus obtained with several histamine-producing organisms are shown in table 2. This table shows that, with two exceptions, the maximum yields of histamine are obtained at pH 5.0 to 5.5. In no case (except with *Aerobacter aerogenes*) was there appreciable histamine formation at a pH more alkaline than 6.2.

TABLE 2

The effect of the pH on the histamine production on medium 5 (meat extract-peptone-histidine, but with 0.3 per cent glucose)

ORGANISM	TEMPER-ATURE OF INCUBATION	pH 4.5	pH 5.0	pH 5.3	pH 5.5	pH 5.7	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
	°C.										
Escherichia coli Sta.	26	0.008	0.016	0.016	0.021	0.037	0.070	0.013	0	0	0
Escherichia coli Ev.	31	0.107	0.168	0.053	0.044	0.032	0.010	0.002	0	0	0
Escherichia coli Har.	37	0.065	0.123	0.098	0.098	0.068	0.020	0.005	0	0	0
Salmonella schottmuelleri Old.	31	0.157	0.389	0.364	0.304	0.276	0.065	0.034	0	0	0
Shigella dysenteriae St.	31	0.357	0.432	0.448	0.245	0.242	0.245	0.020	0	0	0
Shigella paradysenteriae Ryn.	31	0.061	0.133	0.157	0.120	0.088	0.073	0.020	0	0	0
Shigella alkalescens Kau.	31	0.123	0.266	0.197	0.187	0.136	0.081	0.015	0	0	0
Eberthella typhi Mt. S.	37	0.107	0.264	0.357	0.352	0.243	0.165				
Aerobacter aerogenes Hul.	26	0.136	0.211	0.485	0.501	0.488	0.485	0.485	0.488	0.272	0.048

The results are recorded as mgm. of histamine di-hydrochloride per cubic centimeter of culture. The time of incubation was 14 days. The temperatures selected were those that are optimal for histamine production by that organism.

The two organisms in table 2 that gave unusual results are *Escherichia coli* Sta. and *Aerobacter aerogenes* Hul. *E. coli* Sta., which is a very efficient producer of histamine on other media (table 3) has consistently given low values for the meat-extract-peptone-histidine-glucose medium, which apparently contains some inhibitory factor which may also be responsible for the somewhat atypical pH effect. *Aerobacter aerogenes* Hul., and other histamine producers of this species, differ from all other histamine-forming organisms in being uninfluenced by the pH over a wide range. Only at the extremes (pH 5.0 to 5.5, and 7.5 to 8.0) is there any decrease in histamine production.

Koessler and Hanke (1919b) concluded, from their study of

Escherichia coli, that an acid reaction was indispensable for histamine production. Bertrand and Berthelot (1913), Mellanby and Twort (1912–13) and Jones (1918), on the other hand, have described organisms which produce histamine in alkaline reactions only, never in the presence of glucose. A search was made for such organisms, using the technic employed by these authors, but without success. As Jones succeeded only once in 50 attempts, such organisms cannot be common.

III. THE EFFECT OF THE TEMPERATURE AND THE COMPOSITION OF THE MEDIUM ON HISTAMINE PRODUCTION

Table 3 shows the effect of these two factors on histamine production by several representative organisms.

Temperatures above 37°C. depress histamine formation by all of the bacteria tested. In most cases, temperatures of 26°C. or less are also unfavorable. With some organisms (such as *Salmonella enteriditis* V and some not given in table 3) variations between 37°C and 26°C. have very little effect. In most instances, however, the temperature effect is very striking. An extreme case is that of *Escherichia coli* Sta., which produces over 40 times as much histamine at 26°C. as at 37°C. on the ammonium-nitrate-histidine-glucose medium. Differences of 3- or 4-fold in the histamine yield with a variation of only a few degrees of temperature are common.

Different species and even different strains of the same species respond differently to temperature variations. Thus, most strains of *Escherichia coli* have their optimum for histamine production at 31° to 26°C., but others at 37°C. (such as *Escherichia coli* Har., table 3). All of the *Salmonella* strains studied have their optimum at 34° to 31°C.; 7 strains of *Eberthella typhi* at 37° to 34°C.; 4 strains of *Shigella* at 31° to 26°C.; 5 strains of *Aerobacter aerogenes* at 26°C.; *Clostridium welchii* and *Bacteroides varius* at 37° to 34°C.; and *Bacteroides ovatus* at 31°C.

The composition of the culture fluid has a marked effect upon histamine production, as has been recognized by Bertrand and Berthelot (1913) and by Hanke and Koessler (1922). In these

TABLE 3

Effect of the temperature and the composition of the medium on histamine production

ORGANISM	TEMPERATURE	MEDIUM 1. AMMONIUM-NITRATE-HISTIDINE-GLUCOSE	MEDIUM 2. ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 3. ASPARAGINE-HISTIDINE-CYSTEINE-GLUCOSE	MEDIUM 4. YOLK INFUSION-ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 5. MEAT EXTRACT-PEPTONE-HISTIDINE-GLUCOSE	MEDIUM 6. MEAT EXTRACT-PEPTONE-HISTIDINE-GLYCEROL
	°C.						
	41				0.009	0.011	
Escherichia coli Sta.	37	0.010	0.032	0.157	0.055	0.046	0.537
	34	0.185	0.331	0.840	0.480		
	26	0.425	0.451	0.629	0.637	0.126	0.633
	20	0.041	0.440	0.580	0.522	0.078	0.118
	41			0.013	0.025	0.021	
Escherichia coli Har.	37	0.003	0.017	0.058	0.175	0.166	0.033
	31			0.032	0.058	0.141	
	26	0.002	0.001	0.023	0.029	0.110	
	37	0.014	0.104	0.007	0.267	0.124	
Escherichia coli Tem.	31	0.061	0.216	0.065	0.492	0.151	0.041
	26	0.064	0.272	0.065	0.645	0.197	
	37	0.002	0.001	0.014	0.138	0.336	0
Salmonella schott-muelleri Old.	34	0.003	0.005	0.012	0.268	0.288	0
	31	0.003	0.009	0.021	0.264		
	26	0.004	0.011	0.016	0.305	0.240	0
	37	0	0	0.007	0.200	0.135	0
Salmonella enter-iditis V.	34	0	0	0.008	0.211	0.129	0.010
	31	0	0	0.007	0.352	0.236	0.015
	26	0	0	0.009	0.261	0.196	
	41				0.027	0.111	
Eberthella typhi Mt. S.	37				0.298	0.303	0.010
	34		No growth		0.341	0.316	0.008
	31				0.200	0.283	0.005
	26				0.099	0.181	
	37	0	0.007	0.016	0.267	0.234	0.022
Shigella dysen-teriae St.	34			0.026	0.481	0.362	
	31	0	0.034	0.037	0.498	0.403	0.003
	26			0.035	0.391	0.411	

TABLE 3—*Concluded*

ORGANISM	TEMPERATURE	MEDIUM 1, AMMONIUM-NITRATE-HISTIDINE-GLUCOSE	MEDIUM 2, ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 3, ASPARAGINE-HISTIDINE-CYSTEINE-GLUCOSE	MEDIUM 4, YOLK INFUSION-ASPARAGINE-HIS-TIDINE-GLUCOSE	MEDIUM 5, MEAT EX-TRACT-PEPTONE-HIS-TIDINE-GLUCOSE	MEDIUM 6, MEAT EX-TRACT-PEPTONE-HIS-TIDINE-GLYCEROL
	°C.						
Shigella para-dysenteriac Rya.	37	0.013	0.069	0.076	0.560	0.133	0.388
	31	0.080	0.347	0.399	0.587	0.111	0.356
	26	0.102	0.475	0.644	0.651	0.230	
Shigella alkales-cens Kau.	37	0.024	0.049	0.104	0.352	0.060	0.590
	31	0.072		0.544	0.803	0.136	0.451
	26			0.651	0.597	0.206	0.060
Acrobacter aero-gcnes Hul.	41			0	0	0	0
	37	0.001	0.215	0.092	0.011	0.015	0.301
	31	0.025	0.381	0.500	0.512	0.405	0.325
	26	0.042	0.466	0.582	0.619	0.243	0.281
	20	0.003	0.485	0.419	0.275		
Clostridium welchii Ti.	37				0.165*	0.391	
	31				0.157*	0.251	
Bacteroides ovatus	37				0.076*	0.091	
	31				0.296*	0.267	

Clostridium welchii Ti. and *Bacteroides ovatus*: No growth (Media 1, 2, 3).

Results are expressed in milligram of histamine di-hydrochloride per cubic centimeter of culture. The time of incubation was 14 days.

* Cysteine hydrochloride was added to this medium in a concentration of 1:1000.

experiments, the lowest yields usually occurred with the ammonium-nitrate-histidine-glucose medium. This is partly explained by the fact that growth here is often sparse or lacking; the pH attained is usually 5.6 to 6.0, which is not optimal. With this medium, a small quantity of imidazol bases other than histamine may appear in the acid extract (see table 6).

When an organic compound of nitrogen, such as asparagine, is supplied in addition to the histidine, the histamine yield is usually increased. Such an increase was observed by *Hanke* and

Koessler (1922) with several amino acids. This increase is in part due to the more favorable pH obtained (pH 5.0 to 5.5 when glucose is present). In many cases, the addition of cysteine to the asparagine medium still further increases histamine production; with one organism, however (*Escherichia coli*, Tem.), the addition of cysteine greatly diminished the yield.

When egg-yolk infusion was added to the medium, histamine production was in most cases greatly stimulated. In many instances, especially with the anaerobes and with all strains of *Eberthella typhi*, the addition of cysteine still further augmented the yield of histamine.

The meat-extract-peptone-histidine-glucose medium was rather variable in its effect on histamine production. In many cases, it was as good or better than other media; while with other organisms it gave comparatively low yields.

When glycerol was used instead of glucose, as in the meat-extract-peptone-histidine-glycerol medium, the production of histamine was usually very low. This is probably due to the fact that these organisms produce little or no acid from glycerol, hence the pH remains too alkaline for histamine production. But in 5 instances (3 strains of *Escherichia coli*, *Shigella paradysenteriae* Rya., and *Shigella alkalescens* Kau.) the production of histamine was considerably increased when glycerol was substituted for glucose (tables 3 and 4). Also, with *Aerobacter aerogenes*, there was a marked difference in favor of the glycerol, but only at the higher temperatures. With the 5 strains mentioned above, the pH with the glycerol medium ran between 5.7 and 6.2, reactions which one would expect to be less favorable for histamine production than the pH 5.0 to 5.3 obtained on the glucose medium. The results indicate that for these strains at least, the carbohydrate has some other effect than that of stimulating growth and providing a favorable acidity.

The addition of 5 per cent of serum to these media made very little difference in the histamine yield. As a rule, the addition of serum slightly diminished histamine production; in a few cases, it slightly increased the yield.

TABLE 4

Production of histamine by bacteria on media of different composition

ORGANISM	TEMPERATURE	MEDIUM 1, AMMONIUM-NITRATE-HISTIDINE-GLUCOSE	MEDIUM 2, ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 3, ASPARAGINE-CYSTEINE-HISTIDINE-GLUCOSE	MEDIUM 4, YOLK INFUSION-ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 5, MEAT EXTRACT-PEPTONE-HISTIDINE-GLUCOSE	MEDIUM 6, MEAT EXTRACT-PEPTONE-HIS-TIDINE-GLYCEROL
	°C.						
Escherichia coli Sta.*	26	0.425	0.452	0.629	0.637	0.126	0.033
Escherichia coli Har.*	37	0.003	0.017	0.058	0.175	0.166	0.033
Escherichia coli O'L.	26	0.002	0.002	0.003	0.019	0.097	0.005
Escherichia coli Sch..	26	0	0.003	0.012	0.044	0.088	0
Escherichia coli Tem.*	26	0.064	0.272	0.065	0.615	0.197	
Escherichia coli Mal... . .	31	0	0	0	0.025	0.053	0
Escherichia coli Ort.* . .	37	0	0	0	0.002	0.154	0.268
Escherichia coli Kie. .	37	0	0 067	0.106	0.121		
Escherichia coli Bro.*	37	0	0.020	0.016	0.205	0.107	0.120
Escherichia coli Rak. ..	37	0	0.003	0.005	0.027		
Escherichia coli Pen.	26	0	0.002	0.002	0.072	0.273	0.039
Escherichia coli Ev....	26	0	0.001	0.006	0 021	0.188	0.030
Escherichia coli Jon. . .	26	0	0.007	0.006	0.051		
Escherichia coli H 1	34				0.307	0.128	
Escherichia coli H 2	34			0	0 011	0.110	
Escherichia coli Ton. . .	34				0.213		
Escherichia coli McC.	34				0 053		
Escherichia coli Mer. . .	34				0 212		
Salmonella paratyphi St.	31	0	0 002	0 027	0.109		
Salmonella paratyphi Frl.*	31	0	0	0.009	0 267	0.112	0 016
Salmonella schottmuelleri Old.*	34	0 003	0.005	0 012	0 268	0.288	0
Salmonella schottmuelleri 222*	31			0.007	0 352	0.321	0 006
Salmonella schottmuelleri Rak.*	31	0	0	0	0 190		
Salmonella enteriditis V.*	31	0	0	0 007	0 352	0.236	0 015
Salmonella enteriditis McK.	31			0 009	0 101		
*Salmonella suis**	31			0 002	0.147	0 305	0 001
*Salmonella aertrycke**	31			0	0 152	0 203	0 017
Eberthella typhi Pfe.*.	37				0 087	0.151	0
Eberthella typhi Sch.*	37				0 188	0.090	0 005
Eberthella typhi Mt. S.*..	37				0 298	0 303	0 010
Eberthella typhi Mor. .	37	No growth			0 064	0 082	0
Eberthella typhi Spa.	37				0 009	0.115	0
Eberthella typhi Bra. . . .	37				0 007	0 129	0
Eberthella typhi Dan.* . .	37				0 090	0 200	0 002

* Crystals of histamine di-picrate, m p. 238-241°C. were prepared from the acid extracts.

TABLE 4—*Concluded*

ORGANISM	TEMPERATURE	MEDIUM 1. AMMONIUM-NITRATE-HISTIDINE-GLUCOSE	MEDIUM 2. ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 3. ASPARAGINE-CYSTEINE-HISTIDINE-GLUCOSE	MEDIUM 4. YOLK INFUSION-ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 5. MEAT EX-TRACT-PEPTONE-HIS-TIDINE-GLUCOSE	MEDIUM 6. MEAT EX-TRACT-PEPTONE-HIS-TIDINE-GLYCEROL
	°C.						
Shigella dysenteriae St.*.........	31	0	0.034	0.037	0.498	0.403	0.003
Shigella paradysenteriae Rya.*...	26	0.102	0.475	0.644	0.651	0.230	0.356
Shigella paradysenteriae Son.....	26	0	0	0	0.097	0.047	
Shigella alkalescens Kau.*.......	31	0.072		0.544	0.803	0.136	0.451
Aerobacter aerogenes Hul.*.......	26	0.042	0.466	0.582	0.619	0.243	0.284
Aerobacter aerogenes Woo.*.......	26	0.093			0.338	0.668	0.700
Aerobacter aerogenes Sm..........	26				0.041	0.020	0.078
Aerobacter aerogenes Mc..........	26			0.027	0.141	0.184	0.067
Aerobacter aerogenes El..........	31				0.272	0.144	0.034
Clostridium welchii Ti.*.........	37				0.165†	0.391	
Clostridium welchii Eg.	34				0.120†	0.251	
Clostridium welchii Br.	34	No growth			0.120†	0.167	
Clostridium welchii Ca.	34				0.162†	0.275	
Bacteroides ovatus..............	31				0.296†	0.275	
Bacteroides varius..............	37				0.022†	0.256	

† Cysteine hydrochloride was added to a concentration of 1:1000. Results are expressed in milligram of histamine di-hydrochloride per cubic centimeter of culture. The time of incubation was 14 days.

IV. THE ORGANISMS THAT WERE FOUND TO PRODUCE HISTAMINE

In table 4, all of the organisms that were found to produce histamine have been listed. Most of them were tested at several different temperatures; in this table, only the yields for one temperature are given. This was not always the optimum, as the optimum temperature was not determined in every case.

One is impressed by the fact that every organism in table 4 has its habitat in the intestinal tract; not one non-intestinal organism produced histamine. The converse, however, is not true, for many species of intestinal bacteria do not produce histamine.

Every strain of *Escherichia coli* that was tested produced

some histamine. This was likewise true of every strain of the genus *Salmonella* and of every strain of *Eberthella typhi*. On the other hand, of the 8 strains of *Shigella* that were investigated, only the 4 given in table 4 were positive; and of 14 strains of *Aerobacter aerogenes*, only 5 produced histamine. Four strains only of *Clostridium welchii* were studied; all were positive. Only one strain each of *Bacteroides ovatus* and *Bacteroides varius* were available.

V. HISTAMINE PRODUCTION BY "RESTING" BACTERIA

Medium 7, containing only histidine and salts, was employed in these experiments. As little or no growth can take place in

TABLE 5

Histamine production by resting bacteria

ORGANISM	HISTAMINE DI-HY-DROCHLORIDE PRODUCED	HISTIDINE CONVERTED
	mgm. per cc.	*per cent*
Eschericha coli Sta	0.694	85.9
Escherichia coli Har	0	0
Salmonella schottmuelleri 222	0	0
Eberthella typhi Mt. S	0	0
Shigella dysenteriae St	0	0
Shigella paradysenteriae Rya	0.437	54.1
Shigella alkalescens Kau	0.778	96.3
Aerobacter aerogenes Hul	0	0

The temperature was 34°C., and the time of incubation was 7 days.

this fluid, very heavy inoculations were made. Young agar cultures were washed twice with sterile distilled water, and enough organisms were added to the medium to give a density of about 2 billion bacteria per cubic centimeter.

In one experiment (table 5) the medium was brought to pH 5.2 with acetic acid; it is therefore well buffered at this pH.

All of the organisms shown in table 5 will produce abundant histamine on other media. Yet only 3 of these 8 organisms formed histamine under these conditions; the same 3 will form histamine in the ammonium-nitrate-histidine-glucose medium. The 5 negative organisms of table 5 partially converted the

histidine to another base that appeared in the acid extracts; this base does not contain the imidazol nucleus, for only a pale yellow color is produced when it is coupled with sulfanilic acid. It is apparently identical with a base encountered by Koessler and Hanke (1919b) to which they ascribed the formula $HCNH_2:CNH_2CH_2CH_2NH_2$. Imidazol bases other than histamine did not appear in the acid extracts.

VI. THE PRODUCTION OF IMIDAZOL BASES OTHER THAN HISTAMINE

As stated before, imidazol bases other than histamine appeared in the acid extracts only when the cultures were made on medium

TABLE 6

Production of histamine and of extractable imidazol bases other than histamine on the ammonium-nitrate-histidine-glucose medium

ORGANISM	HISTAMINE	IMIDAZOL BASE, NOT HISTAMINE
Escherichia coli Sta............................	0.299	0.025
Escherichia coli Kier...........................	0	0.012
Escherichia coli Tem...........................	0.061	0.012
Shigella paradysenteriae Rya...................	0.057	0.015
Shigella alkalescens Kau.......................	0.072	0.016

The time of incubation was 14 days; the temperature, 31°C.

The results in both columns are expressed as histamine di-hydrochloride in milligrams per cubic centimeter.

1. In this medium, ammonium salts and nitrates are the only sources of nitrogen beside the histidine, and glucose is present. When the amount of these bases is small (as is invariably the case), they will not be precipitated from the acid extracts by phosphotungstic acid (Eggerth, Littwin, and Deutsch, 1939), whereas the histamine will be completely removed. If the precipitate is dissolved in sodium carbonate solution, and the supernatant fluid is neutralized, both fractions can be assayed with the diazo reagent in the usual way. The results of several such tests are given in table 6.

This table shows that the total amounts of non-histamine imidazol bases in the acid extract were always small. For

this reason, no attempt was made to isolate and identify them. When treated with the diazo reagent, their color was redder than that of the histamine standard, suggesting that they might be methyl imidazol or some homologue such as ethyl or vinyl imidazol.

VII. ORGANISMS THAT DO NOT PRODUCE HISTAMINE

The following bacteria were tested on two or more media each, and at two different temperatures, and were found negative for histamine.

Number of strains

Proteus vulgaris	6
Alcaligenes fecalis	2
Shigella paradysenteriae	4
Aerobacter aerogenes	9
Klebsiella pneumoniae	3
Klebsiella ozaenae	1
Klebsiella rhinoscleromatis	1
Vibrio comma	1
Vibrio metchnikovi	1
Brucella abortus	1
Brucella melitensis	1
Pseudomonas aeruginosa	2
Neisseria intracellularis	3
Neisseria gonorrheae	2
Neisseria flava	1
Neisseria catarrhalis	1
Neisseria crassus	1
Streptococcus hemolyticus	3
Streptococcus viridans	1
Enterococcus	6
Diplococcus pneumoniae	3
Staphylococcus aureus	3
Corynebacterium diphtheriae	1
Bacillus subtilis	2
Bacillus mycoides	1
Bacillus mesentericus	1
Bacillus megatherium	1
Lactobacillus acidophilus	2
Clostridium tetani	2
Clostridium putrificum	1
Clostridium histolyticum	1
Clostridium bifermentans	1
Bacteroides bifidus	4
Bacteroides pseudoramosus	3

Bacteroides acrofaciens.. 3
Bacteroides biformis... 3
Bacteroides avidus... 2
Bacteroides limosus.. 1
Bacteroides catenaformis... 2
Bacteroides lentus... 3
Bacteroides gulosus.. 3
Bacteroides thetaiotaomicron... 3
Bacteroides variabils.. 3
Bacteroides uniformis.. 3
Bacteroides vulgatus... 4
Bacteroides distasonis... 3
Bacteroides exiguus.. 1
Bacteroides vescus... 1
Bacteroides insolitus.. 1

Where organisms did not grow well on the usual media, special media were used. Thus, 5 per cent of serum was added to cultures of the *Neisseria*, *Streptococcus*, and *Diplococcus* genera; and *Lactobacillus acidophilus* was cultivated in a milk histidine medium.

DISCUSSION

The only other investigations in which a large number of strains and species of organisms were studied have been those of Hanke and Koessler (1922) and of Koessler, Hanke, and Sheppard (1928). Hanke and Koessler studied 62 strains (29 of these were *Escherichia coli*) on a medium containing only histidine, inorganic salts, and glycerol. Six of these strains—all of them *Escherichia coli*—formed histamine. No positive results were obtained with any member of the *Salmonella*, *Eberthella*, or *Shigella* genera. Koessler, Hanke, and Sheppard studied 223 strains from 94 species on a medium containing meat extract, peptone, histidine, whole blood, and glycerol. (These authors preferred glycerol to glucose because, when glucose was used, troublesome interfering substances were extracted by their method.) In this investigation, 9 histamine producing organisms were found; 2 were *Escherichia coli*, 1 was a *Salmonella enteriditis*, 1 was a *Salmonella schottmuelleri*, and 5 were *Salmonella morgani*. In the same series, 7 other strains of *Escherichia coli* and 42

other strains of *Salmonella* were negative, as were also all strains of *Eberthella, Shigella,* and *Aerobacter.*

In the present investigation, histamine was formed by every one of the 18 strains of *Escherichia coli* tested; likewise every one of 9 strains of *Salmonella* (from 5 species), and every one of 7 strains of *Eberthella typhi.* Four out of 8 strains of *Shigella* were positive, as well as 5 out of 14 strains of *Aerobacter acrogenes.* Organisms from all of these genera were active producers of histamine, often converting from 30 to 100 per cent of the histidine to the amine. There can be no doubt that the color-producing substance formed by these organisms actually was histamine, as the crystalline di-picrates were prepared in most cases from the acid extracts (table 4).

There are several reasons why many more histamine-positive organisms were found than in the above mentioned investigations. The most important one is that glucose was employed in the media instead of glycerol. When glucose is supplied, the favorable pH of 5.0 to 5.5 is usually obtained, whereas most organisms do not produce enough acid from glycerol to permit histamine formation. Tables 3 and 4 show the striking effect of substituting glucose for glycerol.

Very often the temperature of incubation is an important contributory factor. Hanke and Koessler (1922) and Koessler, Hanke, and Sheppard (1928) incubated their cultures at 37.5°C., which is not, in most cases, the optimal temperature for histamine production.

Hanke and Koessler (1922) employed a medium that contained no organic source of nitrogen except histidine itself. Tables 3 and 4 show that such a medium is usually unfavorable to histamine production, and that very few organisms will be positive when it is used.

SUMMARY

1. Using a simplified technic previously described (Eggerth, Littwin, and Deutsch, 1939) histamine production has been determined in cultures of 49 strains of bacteria, belonging to 14 species of 7 genera. These organisms all have their habitat in the intestinal tract.

2. With the exception of *Clostridium welchii*, all of these organisms require free histidine for histamine formation.

3. In a favorable medium, histamine production begins within 24 hours and continues rapidly for 4 to 5 days, after which the rate of production decreases.

4. For most organisms, the optimal pH for histamine production is pH 5.0 to 5.5, and no histamine is produced at reactions more alkaline than pH 6.5. However, the histamine forming strains of *Aerobacter aerogenes* will produce this amine at any pH between 5.0 and 8.0.

5. The temperature of incubation markedly affects histamine formation. Temperatures higher than 37°C. and lower than 26°C. are usually unfavorable. Between these limits, the optimal temperature varies a great deal with different organisms.

6. The yield of histamine is determined also by the composition of the culture medium. Where only inorganic compounds of nitrogen are supplied, in addition to histidine, the yields of histamine are usually low, and some of the histidine may be converted to other imidazol bases. The addition of amino acids, such as asparagine and cysteine, or of peptone, or of egg yolk or meat infusion, increases histamine production. The nature of the added carbohydrate is also important, chiefly because of the effect of the pH of the culture.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CENTRAL NEW YORK STATE BRANCH

VETERINARY COLLEGE, CORNELL UNIVERSITY, NOVEMBER 19, 1938

THE SEVENTH EDITION OF STANDARD METHODS FOR THE EXAMINATION OF DAIRY PRODUCTS. *Robert S. Breed,* N. Y. State Agricultural Experiment Station, Geneva.

This well-known report is soon to be issued in a new edition as approved by the American Public Health Association at their Kansas City meeting, October 24–28, 1938. The changes affect the composition of the agar used in making agar plate counts, and the temperature of incubation used for these plates. It is required that properly constructed incubation chambers be used and that the temperature be held at a point where it does not exceed 37°C nor fall lower than 35°C.

The use of microscopic examination of pasteurized milk as a means of checking the presence of large numbers of bacteria, either living or dead is also emphasized in this new report.

THE UDDER STREPTOCOCCI OF EIGHTEEN DAIRY HERDS. *Jean Ferguson,* Veterinary College, Cornell University, Ithaca.

A mastitis-survey of 18 dairy herds has included the examination of quarter milk samples for the presence of streptococci. For this purpose 0.1 cc. of milk was plated on 8 per cent horse-blood agar. One hundred thirty-eight (21 per cent) of the 655 cows were infected with streptococci. Of the 229 cultures isolated from as many quar-

ters, 160 (70 per cent) were *Streptococcus agalactiae;* 16 (7.0 per cent) were *Streptococcus dysgalactiae;* 25 (10.8 per cent) were *Streptococcus uberis;* 13 (5.6 per cent) were of other types, and on the remaining 15 cultures (6.5 per cent) no tests were made.

All of the *Streptococcus uberis* and *Streptococcus dysgalactiae,* 52 of the *Streptococcus agalactiae* cultures, and 11 of the 13 streptococci of other types produced green discoloration of blood agar. The remainder of the *Streptococcus agalactiae* cultures were either indifferent to blood or produced narrow- or broad-zone hemolysis. This broad-zone hemolysis was distinguished from the hemolysis of two Group C and E cultures by an outer zone of hemolysis.

In those herds in which *Streptococcus agalactiae* was present, it was the predominating organism and the infection tended to be widespread. Infections due to other streptococci appeared to be sporadic in origin.

Only in the most heavily infected herd could streptococci have been observed in a Breed smear of the mixed milk.

FOUR TEMPERATURE OPTIMA OF BACTERIA. *Leslie Dorn,* N. Y. State College of Agriculture, Ithaca.

THE INHIBITION OF BACTERIOPHAGE BY PHOSPHOLIPIDS. *Clara H. Williams,*

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Leslie A. Sandholzer and George Packer Berry, University of Rochester, School of Medicine and Dentistry, Rochester.

Phospholipids, from bacterial and non-bacterial sources, and cholesterol were tested for an inhibitory action on a bacteriophage (C13, Burnet) which lyses a variety of coliform organisms. The plaque-count method in semi-solid agar was employed.

Using two bacteriophage-susceptible strains of *Escherichia communior* as test organisms, it was found that lecithin from soybeans and sphingomyelin from sheep brains failed to inhibit the bacteriophage in concentrations up to 1 per cent. However, two samples of cephalin, one from beef livers and the other from sheep brains, were markedly inhibitory in concentrations of 0.2 per cent. This action persisted after the lecithin had been mixed with the samples of cephalin in a proportion of 5:1.

Phospholipids from the blood plasma of a dog exerted no inhibition. A sample of purified cholesterol, however, was strongly inhibitory in a concentration of 1 per cent.

The two strains of *Escherichia communior*, mentioned above, and a strain of *Escherichia coli* which was resistant to the bacteriophage, were used as source materials for the preparation of bacterial phospholipids. The phospholipids from all three strains were markedly inhibitory, a finding which indicates that the inhibitory activity is not specific in regard to susceptibility or resistance to bacteriophagy.

FURTHER EXPERIMENTS IN THE DISINFECTION OF SEEDS. *A. W. Hofer and H. C. Hamilton*, New York State Agricultural Experiment Station, Geneva.

Further studies have been made to discover a disinfectant selective for the contaminating organisms of seeds.

Among the agents used, two were unusual. One was the commonly used chlorine, but in the form of hypochlorous acid; the other was a war gas, iodoacetone, selected for its penetrating properties. (Chlorine is ordinarily used as a hypochlorite; when this is treated with a weak acid, hypochlorous acid is liberated—an agent many times more potent than the relatively stable commercial preparations. Iodoacetone is prepared from chloracetone and is water soluble.)

Many experiments were made in this research to improve methods. Seeds were soaked in water before applying the disinfectant. In other experiments the coats were removed from peas and soybeans to obtain a clue to the location of contaminants. It required in both cases stronger solutions to obtain results equivalent to those for seeds not previously soaked. Seeds, apparently disinfected, were crushed and returned to the beef broth where bacterial growth subsequently occurred in almost every tube. Evidently the bacteria were in the interior of the seed.

CONTAMINATION OF DRINKING GLASSES BY STREPTOCOCCI. *Leo A. Dick*, New York State Agricultural Experiment Station, Geneva.

Numerous workers have reported data which show that eating utensils are important in the transfer of respiratory and saliva-borne infections.

Since the basic danger lies in the incomplete removal of consumer-contamination, the present study has been conducted to ascertain the validity of employing *Streptococcus salivarius* as an indicator-organism of mouth contamination. The closed lips of 100 individuals were wiped with sterile cotton swabs. To determine whether the organisms from the lips and mouth are deposited on drinking glasses, each

individual was given a drink of sterile fruit juice from a sterile glass. The rim of the glass was then swabbed with a sterile swab. The swabs were incubated for 12 hours at 38°C. in enrichment broth and then plated in 8 per cent horse-blood veal infusion agar and incubated for 24 hours at 37°C. Typical streptococcal colonies exhibiting *alpha* to *gamma* hemolysis were picked and their fermentative reactions studied.

Streptococcus salivarius was recovered from the lips and drinking glass of every individual.

THE ETIOLOGY OF TUBERCULOSIS OF FISH. *J. A. Baker*, N. Y. State Veterinary College, Ithaca.

ON THE NATURE OF ADAPTIVE ENZYMES. *Otto Rahn*, N. Y. State College of Agriculture, Ithaca.

SHOCK DISEASE. *Carl L. Larson*, University of Rochester, Rochester.

A STRAIN OF ENTEROCOCCUS (STREPTOCOCCUS FECALIS) GIVING CROSS-REACTIONS WITH PNEUMOCOCCUS TYPE III ANTISERUM, ISOLATED FROM THE BLOOD STREAM OF A PATIENT. *E. Witebsky, J. MacCallum, E. Neter and C. Richbart*, The Buffalo General Hospital, Buffalo.

The strain of enterococcus under investigation was isolated from a patient with septicemia following abortion. It grew well on blood agar plates, causing greenish discoloration. It produced in 1 per cent glucose broth a pH of 4.5; it caused acid production, reduction, and clot formation in litmus milk, reduced methylene blue, fermented aesculin, produced acid from lactose, salicin, mannitol. It grew in 6.5 per cent NaCl-broth, was bile-insoluble, and grew in broth containing optochin in a concentration of 1:100,000. It was relatively heat-resistant. The supernate of the broth culture gave a definite reaction with Pneumococcus Type III antiserum, although the precipitation occurred somewhat more slowly and was somewhat less intensive than with Pneumococcus Type III. The suspension of the strain was agglutinated by Pneumococcus Type III antiserum. Although the strain failed to kill mice upon intraperitoneal injection, it could be recovered from the heart's blood. Smears of the peritoneal exudate revealed encapsulated diplococci; the supernate giving precipitation with Pneumococcus Type III serum. Other pneumococcal antisera (including Type VIII) failed to give any reaction with either the culture or the peritoneal exudate. The cross reaction with this particular strain was constantly found for about ten weeks; subsequent subcultures, however, lacked this property.

THE ACTION OF SULFANILAMIDE ON MENINGOCOCCI IN VIVO AND IN VITRO. *Erwin Neter*, Children's Hospital and University of Buffalo, Buffalo.

At the Children's Hospital two cases of meningococcal meningitis were treated with sulfanilamide alone. One patient was a baby ten weeks old who was suffering from a recrudescence of meningococcal meningitis. After the intraspinal injection of sulfanilamide, the spinal fluid became sterile in 72 hours and the patient made an uneventful recovery. The spinal fluid of the second patient became sterile 24 hours after the administration of large doses of sulfanilamide by mouth.

The action of the sulfanilamide upon meningococci in the spinal fluid from patients with meningococcal meningitis was studied. It was found (1) that sulfanilamide inhibited the growth of meningococci in spinal fluid; (2) that

previous treatment of meningococci with sulfanilamide was followed by a retardation of the growth rate on subculture *or by a loss of viability.* These effects of sulfanilamide were also obtained in the absence of leucocytes.

THE BACTERIOSTATIC EFFECT OF SULFANILAMIDE, DISULON, AND NEOPRONTOSIL IN VITRO ON ACID-FAST BACILLI. *C. M. Carpenter and L. L. Gibbons,* University of Rochester School of Medicine and Dentistry, Rochester.

Thirty strains of acid-fast bacilli were exposed *in vitro* to 1:10,000 concentrations of sulfanilamide, Disulon, and Neoprontosil. This procedure was repeated at least 5 times with each strain. A number of the strains were exposed in the same manner to 1:5,000 and 1:2,500 concentrations of each compound.

Twenty-one of the strains were chromogenic. Fourteen were isolated from cases of cutaneous leprosy, and 7 from miscellaneous sources. Of the 9 non-chromogenic strains, 5 were from lepromata, and 1 from sputum; 3 were *Mycobacterium tuberculosis*, avian and human strains.

Exposure to sulfanilamide and to Disulon inhibited completely or partially the growth of 8 strains of so-called *Mycobacterium leprae*, 1 strain of *Mycobacterium leprae-murium,* and 1 unidentified strain isolated from sputum. Of these leprosy strains, 4 were isolated by Kedrowski, 3 in our laboratory, and 1 by Needham. No inhibition was noted in the growth of the strains of *Mycobacterium tuberculosis,* the so-called saprophytic chromogenic strains, and the 6 remaining cultures isslated from lepromata.

Concentrations of the drugs greater than 1:10,000 showed no further in-

hibition. Neoprontosil did not inhibit growth in any instance. No bactericidal effects were observed. The action of sulfanilamide and Disulon was entirely bacteriostatic.

RESISTANCE OF THE GONOCOCCUS IN VITRO TO GRADUALLY INCREASED CONCENTRATIONS OF SULFANILAMIDE. *R. A. Boak and C. M. Carpenter,* University of Rochester School of Medicine and Dentistry, Rochester.

Six strains of *Neisseria gonorrhoeae* were grown in blood-glucose-ascitic fluid broth containing gradually increased concentrations of sulfanilamide and tested daily for viability.

Two strains grew well in 0.01 per cent sulfanilamide on isolation, but failed to survive after 72 hours in 0.02 per cent. After daily transfers for 17 and 12 days, respectively, in 0.01 per cent sulfanilamide, both strains thrived in a 0.02 per cent concentration.

Three strains, which survived for only 24 hours in 0.02 per cent sulfanilamide, were grown for 35 days in 0.01 per cent and then exposed to concentrations of 0.02, 0.03, 0.04, 0.05, and 0.06 per cent of the compound. Each continued to grow in the first three concentrations. In 0.05 and 0.06 per cent, a few organisms from 2 strains remained viable for only 24 hours, while the third strain did not survive.

The sixth strain, recovered from a patient after extensive sulfanilamide treatment, grew luxuriantly in 0.04 per cent when isolated. It required cultivation in 0.02 per cent for 91 days, with daily transfer, before it grew satisfactorily in 0.08 per cent sulfanilamide. A control culture, cultivated without sulfanilamide for the same period of time, did not survive 0.08 per cent for 24 hours.

EASTERN NEW YORK BRANCH

DIVISION OF LABORATORIES AND RESEARCH, STATE DEPARTMENT OF HEALTH
DECEMBER 2, 1938

SOME EFFECTS OF LOW VELOCITY ELECTRONS ON MICROÖRGANISMS. *Caryl P. Haskins*, Union College, Schenectady, New York.

An instrument has been constructed to study the effects of beams of low-velocity electrons of high homogeneity in density and velocity upon various microörganisms. All work is of necessity done in high vacuum. The work is of interest for a number of reasons. The beam acts essentially as a blunt-edged probe wherewith to explore the organism. It may be hoped to shed some light on the quantitative nature of the action of x-rays on living single cells, since the absorbed electrons can be made equal in number and range to the average range and density of the released secondary electrons in tissue when bombarded by x-rays of any desired wavelength over a wide range. It can also be used in investigations of the killing "sensitive volume" concept, and in a study of the mechanism of mutations under ionizing radiations. Work so far has been done with spores of Ascomycete fungi of the genera *Penicillium* and *Aspergillus*, which show good resistance to vacuum killing. Some mutations have been produced in *Aspergillus*, which are shown. It is expected to extend the work to both larger and smaller single-celled organisms, and to viruses.

A STUDY OF PNEUMOCOCCUS TYPE DIFFERENTIATION IN A GENERAL HOSPITAL. *Arthur W. Wright*. Department of Bacteriology, Albany Medical College, Union University, Albany, New York.

During a one-year period, 562 specimens of sputum for pneumococcus type differentiation were received in the laboratory of the Albany Hospital. These specimens came from 300 patients whose ages varied from 17 months to 79 years. Clinically, 93 of the patients suffered from lobar pneumonia, 96 from bronchopneumonia, 2 from empyema, one from meningitis, while 108 had no clinical evidence of pneumonia.

Lobar pneumonia was most common in the fourth, fifth and sixth decades, 49 of the 93 cases falling into these age groups. The predominating pneumococcus types found were I, III, IV, VIII, XII, V and XIV, in that order. These strains were incitants in 62 cases. In 10 cases pneumococci could not be found but other microörganisms, chiefly *Streptococcus hemolyticus*, were isolated. These may have been the inciting agents. Forty-four patients with pneumococcus lobar pneumonia were treated with specific antiserum. This group is too small to use as a basis for an analysis of results.

Of the patients with pneumococcus lobar pneumonia 17 had septicemia, as follows: Type I, 3 cases; Type III, 4 cases; Type XII, 4 cases; Type VIII, 2 cases; and Type IV, V, IX and XIV, one case each. Serum treatment was administered to the patients with Type I and Type V pneumococcus pneumonia and to one of the patients with Type XII pneumococcus infection. Two patients, one who had received Type V antiserum and the other Type XII antiserum, recovered. All others died.

A STUDY OF THE APPEARANCE OF SERUM AGGLUTININS AND POSITIVE S.S.S. SKIN REACTIONS IN PATIENTS WITH

PNEUMOCOCCUS PNEUMONIA—A PRE-
LIMINARY REPORT. *Mabel S. Ingalls,*
Department of Bacteriology, Al-
bany Medical College, Union Univer-
sity, Albany, New York.

Sixteen of seventeen serum-treated
pneumonia cases showed a strong titer
of agglutinins in their blood, after
administration of antiserum. The
seventeenth showed no agglutinins and
died. Of the 16 with agglutinins, 3
also died. On autopsy all 3 were
found to have had complications not
due to pneumococci.

Nine of the above cases were also
tested for skin reactivity to the carbo-
hydrate of the causative pneumococci.
Seven gave positive reactions, one was
negative, despite a strong agglutina-
tion titer and died, and one never gave
a frankly positive reaction, 'lost his
agglutinin titer after 4 days and ran a
temperature of 103°.

The tests for the presence of aggluti-
nin in the patient's serum were carried
out by the slide agglutination method
of Bullowa.[1] The tests for skin re-
activity to the specific carbohydrate
were carried out, according to the
technique of Francis,[2] with purified
carbohydrates from the Division of
Laboratories and Research of the N. Y.
State Department of Health.

From these few results it would seem
the agglutination test is of value in
indicating the concentration of anti-
bodies in the patient's blood.

The carbohydrate skin reaction is
valuable in showing there are no serious
complications and predicting recovery.

A PRELIMINARY REPORT ON THE
CHEMOTHERAPEUTIC ACTION OF 2-
(P-AMINOBENZENESULFONAMIDO)PY-

[1] N. Y. State Jour. Medicine, 37,
Apr. 15, 1937.

[2] J. Ex. Medicine, 57, 4, 617, 1933.

RIDINE, M. & B. 693, ON TYPE-III
PNEUMOCOCCAL INFECTIONS IN MICE
John K. Miller, Division of Labora-
tories and Research, New York State
Department of Health, Albany.

The blood concentration attained
one and two hours after oral adminis-
tration of 10-, 20-, or 40-mg. doses of
M. & B. 693 to 20-gram mice was the
same—from 22.5 to 25 mg. per cent of
free M. & B. 693. Subcutaneous doses
of 20 and 40 mg. produced a concentra-
tion of 10 and 20 mg. per cent, respec-
tively. The free M. & B. 693 in the
viscera was, in general, from 75 to 90
per cent of the blood concentration, but
in the liver only 25 per cent.

The effect of M. & B. 693 and of M. &
B. 693 combined with serum therapy on
pneumococcal infection in 20-gram
mice was studied with 10,000 M.L.D. of
a stock mouse-passage strain of type-
III pneumococcus. Ten-, 20-, or 40-
mg. subcutaneous doses of M. & B.
693 alone or with one dose of specific
antiserum that by itself afforded 20-
per-cent survival were given imme-
diately and at 7, 24, 48, 72, and 96
hours after inoculation of pneumococci
to groups of ten mice. The combined
therapy was definitely the more effec-
tive.

EFFECT OF KETENE ON DIPHTHERIC
TOXIN. *L. W. Hyman,* Division of
Laboratories and Research, New
York State Department of Health,
Albany.

The study of the effect of ketene on
crude diphtheric toxin was undertaken
to ascertain the extent of acetylation
of the amino group and its possible
significance in the formation of toxoid.
The ketene was prepared according to
the method of Hurd and Cochran
(1923). To 100 ml. of toxin, 45 gram.
of sodium acetate were added and the
mixture cooled to 5°C. On introdu-

ing ketene, the pH was lowered; normal sodium hydroxide was added during the acetylation to maintain the desired pH. The solutions were dialyzed against running water and then either adsorbed on calcium phosphate and dissolved in ammonium citrate or concentrated by ultrafiltration. The acetylations were done at pH 7.0, 8.0, and 8.2. The reduction of amino nitrogen ranged from 40 to 100 per cent; with a reduction of over 50 per cent, the Lf value and the number of M.R.D. per milliliter decreased. The acetylated toxins had little or no antigenicity. One toxin which had 1,000,000 M.R.D. per milliliter and an Lf of 18.8 in twenty-six minutes was treated with ketene at pH 8.0 for one hour. The amino nitrogen was reduced 93 per cent, the number of M.R.D. to 500 per milliliter, and the Lf to 13.4, with the time of flocculation increased to four hours.

THE EFFECT OF COLCHICINE ON TISSUE CULTURES OF TADPOLE HEART. *Myrtle Shaw*, Division of Laboratories and Research, New York State Department of Health, Albany.

Although colchicine has been reported as relatively nontoxic for frogs, tadpole cardiac tissue proved susceptible. Fragments failed to show migration and growth when explanted into a medium containing colchicine in a concentration of 1 part per 10,000,000. A concentration of 1:100,000,000 did not inhibit growth.

When one loopful of colchicine, diluted 1:50,000 in Tyrode's solution, was allowed to act for from six to eight hours on cultures in which growth had become established, no mitoses were seen in stages beyond metaphase. Many mitoses were abnormal. A marked increase was noted in the number of dividing cells in comparison with untreated control cultures.

A comparable accumulation of mitoses was seen in cultures treated with diphtheric toxin for twenty-four hours before the application of colchicine. This suggests that the preliminary treatment with toxin had not caused a cessation of cellular activity.

A COLLODION SAC FOR USE IN ANIMAL EXPERIMENTATION *Albert H Harris*, Division of Laboratories and Research, New York State Department of Health, Albany

The sacs described represent a radical departure from those of Gates (1921) although many of his technical details have been adopted. Stainless steel-wire gauze curved as a cylinder is closed at both ends with molded carnauba wax and a glass tube is passed through one end. Both ends of the sac and the line of approximation of the gauze are covered with alcohol-ether collodion which is allowed to dry for several hours. The sac is dipped in the percentage of acetic-acid collodion required in the given experiment, allowed to drain for a specified time (the collodion which collects at the bottom on the inside is aspirated), and then plunged into filtered tap water. All of the solvent is washed out in periodic changes of the water. The rate of flow of water is measured. The sac is sterilized for about eighteen hours in a solution of 1 part of B.K. (hypochlorite solution) in 100 parts of distilled water and then washed in a large volume of sterile distilled water. The sac is filled by a Pasteur pipette and sealed with paraffin and collodion. At the completion of the *in vivo* experiment, the sac is opened by thrusting a hot wire through the collodion and paraffin seal, and the contents are removed by a Pasteur pipette.

OHIO BRANCH

December 3, 1938

A Simplified Apparatus for the Preservation of Bacterial Cultures in the Dried State. *Frank J. Grabill and Merlin L. Cooper*, Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati.

A simple vacuum apparatus was designed to freeze and dry 32 bacterial cultures simultaneously. The apparatus consists of a vacuum pump, two Wolff bottles, attachments for 32 glass ampules of culture and a chemical desiccant, "Drierite."

The principle involved is that of reducing the vapor pressure of the system to the extent that the moisture will evaporate from the surface of the bacterial cultures with sufficient rapidity to freeze them. Once frozen, the water sublimes from the frozen culture to the desiccant, the rate being sufficient to retain the cultures in the frozen state throughout the desiccation period. The ampules containing the bacterial cultures are blown from ordinary 9 mm. glass tubing. After the dehydration of the cultures, the ampules while still attached to the vacuum apparatus are sealed with a suitable blow torch.

All members of the *Salmonella, Eberthella* and *Shigella* groups so preserved have retained their viability and cultural and biochemical characteristics, without any detectable changes, over a period of six months. Further studies and observations are in progress.

The simplicity of the apparatus makes low-temperature desiccation of heat-labile substances available to the average laboratory.

An Accessory Factor Required for the Synthesis of Polysaccharide by Leuconostoc. *Warner Carlson[1] and G. L. Stahly*, Department of Bacteriology, Ohio State University, Columbus.

Studies were made of the mode of action and chemical nature of the raw sugar extract reported by Carruthers and Cooper as stimulating the synthesis of the polysaccharide, dextran, by *Leuconostoc mesenteroides* and *L. dextranicum* in media containing chemically pure sucrose.

Determinations of bacterial numbers, sucrose titers, dextran yields and hydrogen-ion changes in C. P. Sucrose media with and without added raw sugar extract indicated that the stimulating material should be referred to as an "accessory" rather than a "growth" factor, since it caused a marked stimulation of the rate of dextran synthesis without a corresponding significant increase in the observed number of bacteria per cc. Three types of *Leuconostoc* could be distinguished on the basis of ability to synthesize dextran in a medium containing C. P. sucrose, phosphate and peptone: (a) type 1, which did not require the presence of the factor to form approximately the theoretical amount (50 per cent of the sucrose in the medium) of dextran in 48 to 72 hours time; (b) type 2, which could also synthesize the maximum amount of dextran in the absence of any added factor, but only after about 10 days incubation; and (c) type 3, which gave yields of dextran of only a few per cent of the

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[1] H. A. Toulmin, Jr. Research Fellow.

theoretical, regardless of the length of the incubation period. Addition of the factor to the medium allowed all three types to form approximately the theoretical amount of dextran in 24 to 36 hours.

The factor was extracted by alcohol from some unrefined cane sugars, but it was not found in any of the unrefined beet sugars tested. The accessory factor was also demonstrated in the sterile, cell-free filtrates of broth cultures of some strains of *Staphylococcus aureus*, and of C. P. sucrose cultures of type 1 *Leuconostoc*.

Tests on highly active alcoholic extracts of raw sugar demonstrated that the factor is: (1) not a protein; (2) soluble in methyl alcohol, ethyl alcohol and pyridine; (3) insoluble in acetone, ether, dioxane, benzene and ethyl acetate; (4) not adsorbed by activated charcoal; (5) adsorbed by aluminum gels at pH 10, and eluted at approximately pH 4; and (6) resistant to 2 hours autoclaving at 120°C. at pH 4.2, but destroyed under the same conditions at pH 8.5.

DILUTED AND UNDILUTED DIPHTHERIA TOXOID AS IMMUNIZING AGENTS IN MAN. *Hortense Schmitz*, Department of Hygiene and Bacteriology, Western Reserve University, Cleveland.

A COMPARATIVE STUDY OF BACTERIOLOGICAL EXAMINATIONS OF RESTAURANT UTENSILS. *Otto P. Behrer and Wm. M. Moody*, Cincinnati Department of Health, Cincinnati.

Three bacteriological methods of testing restaurant eating utensils are described and evaluated: the direct-culture, moist-swab and the wet-swab methods proposed by Fellers, Levine and Harvey.   The direct-culture method consisted in collecting knife,

fork, spoon, glass and plate in sterile towels and bringing them into the laboratory. Forks, spoons and knives were immersed in melted nutrient agar in sterile petri dishes. The rim of the glass was also immersed in agar and the inside washed with 10 cc. of sterile water of which 1 cc. was plated. The same procedure of washing and plating was followed with the plates. The moist-swab method consists in adding 0.4 cc. of water to the tube containing the swab and autoclaving. In practice the swab is rubbed over the surface to be examined, replaced in the tube and submitted to the laboratory. It is now transferred to a tube of sterile water (2.5 cc.) vigorously shaken and the entire liquid plated in melted agar. The wet-swab method uses 1 tube containing the 2.5 cc. of sterile water with swab, and is used in the same manner as the moist-swab.

In actual practice the moist-swab method was found more practical and is recommended as the method of choice, both because of its adaptability to field service and because of the results obtained.

HETEROPHILE ANTIGENICITY OF BRUCELLA ABORTUS. *D. Frank Holtman*, Department of Bacteriology, Ohio State University, Columbus.

Previous investigators have considered *Brucella abortus* to be free of Forssman heterophile antigen. However, evidence obtained during the present experimentation shows that certain strains of this bacterium can stimulate in rabbits the formation of antibodies capable of agglutinating or hemolyzing sheep red blood cells. Two cultures of *B. abortus*, isolated from supraspinous bursitis in horses, possessed this property even after 100 transfers on an agar medium free of heterophile antigen. Two cultures iso-

lated from cattle failed to demonstrate heterospecificity, although a third culture of bovine origin, after several years of growth on horse serum agar, was able to induce heterophile antibody formation in rabbits. Since the horse provides a rich source of Forssman antigen, an agent lacking in cattle, it seems probable that the heterospecificity of the bacteria was acquired, not only from the living animal, but also from culture media incorporating its serum.

SYSTEMIC TORULOSIS WITH CASE REPORT AND WITH REFERENCE TO EARLY CASES SIMULATING HODGKIN'S DISEASE. *Thomas L. Ramsey*, St. Vincent's Hospital, Toledo.

THE SPECIFICITY OF THE KERATINS. *L. Pillemer and E. E. Ecker*, Institute of Pathology, Western Reserve University, Cleveland.

A BACTERIOLOGICAL AND SEROLOGICAL STUDY OF 245 INFANTS AND YOUNG CHILDREN WITH ACUTE GASTROENTERITIS. *Merlin L. Cooper*, with the assistance of *H. M. Keller, J. B. Milliken, B. Johnson, H. F. Marsh, F. J. Grabill and G. W. Thomas*, Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati.

During the summer of 1938, 796 stools from 245 infants and young children with acute diarrhea were cultured for *Shigella paradysenteriae*. Each stool was streaked on four plates each of Endo's medium, eosin methylene-blue medium, sodium desoxycholate citrate medium and MacConkey's medium. Suspicious colonies were picked and their cultural characteristics determined in suitable carbohydrate media, gelatin and citrate agar. Motility observations were made after 4 and 24 hours' incubation. Forty-one per cent of these patients were found to have *Shigella paradysenteriae* in one or more of their stools.

In the order of decreasing efficiency the four media were as follows: sodium desoxycholate citrate, MacConkey's, eosin methylene-blue, and Endo's.

Three different fermentative types of *Shigella paradysenteriae* were isolated, differing in carbohydrate reactions.

The blood sera of 70 patients were tested for agglutinins for antigens prepared from *Shigella paradysenteriae* isolated from earlier patients and 22 were found positive, 12 slightly positive, and 36 negative.

Agglutinating antisera had also been prepared prior to this summer for 4 of our 7 antigens. The *Shigella paradysenteriae* isolated from 90 of our patients during this study were tested for their agglutinability by these sera. Fifty-five were agglutinated significantly, 12 were doubtful, and 22 were negative.

FURTHER EXPERIMENTS ON THE WASHING OF HEMOPHILUS PERTUSSIS VACCINE. *J. A. Toomey and William Takacs*, Department of Pediatrics, Western Reserve University, Cleveland.

THE GROWTH REQUIREMENTS OF BACILLUS ANTHRACIS. *Maurice Landy*, Research Division, S.M.A. Corporation, Cleveland.

It has been found that, although Knight observed that his "staphylococcus factor" of yeast stimulated growth of *Bacillus anthracis* and subsequently discovered that in his yeast concentrate nicotinic acid and thiamin were the biological factors essential for the growth of *Staphylococcus aureus*,

it is not these two vitamins but still another factor in yeast that is essential for the growth of *B. anthracis.*

The *B. anthracis* growth factor is not replaced by thiamin, nicotinic amide, beta alanine, pimelic acid, glutathione, betaine, riboflavin, glucosamine, vitamin B_6, or the acidic ether extract of yeast, either singly or in various combinations.

Meat extract, liver extract and yeast are good sources of the growth factor.

Two concentrates of the *B. anthracis* factor prepared from yeast exhibited growth stimulation at levels of 1 and 30 micrograms, respectively.

High vacuum distillation of a potent concentrate at temperatures from 120° to 160°C. at 0.002 mm. mercury results in the activity remaining in the residue.

The growth factor is resistant to autoclaving, to acid and alkali, and to light, is adsorbed on norite charcoal at pH 1.4, is soluble in water and aqueous alcohol, and is insoluble in acetone, ether and chloroform.

CENTRAL PENNSYLVANIA BRANCH

State College, Pa., November 26, 1938

Comparative Study of the Intracutaneous Injection of P.P.D. and the Vollmer Patch Test for the Detection of Tuberculosis. *John W. Rice,* Bucknell University.

Due to reluctance on the part of many college students to submit to the use of the hypodermic needle, it was decided to study the patch test devised by Vollmer for the testing of young children.

A group of 265 students were tested by intracutaneous injection of P.P.D. in standard dose and simultaneously with the Vollmer Patch Test. To summarize, 52.8% of the group were negative to both tests; 23% were negative to the Vollmer test but showed a one plus reaction to P.P.D.; 13.6% showed agreement in one plus reactions while in the group of fairly strong reactions to P.P.D., 0.75% were negative; 2.64% were faintly positive; and 1.6% were definitely positive to the Vollmer test. A total of 5.3% showed stronger reactions to the Vollmer test than to ˙P.P.D. Delayed reactions were quite common in the case of the Vollmer test.

The relative high number of negative reactions together with the delayed response of the Vollmer Patch Test would seem to indicate that this test is not applicable to the college age group.

Studies of Cacao Fermentation. *S. Hoynak, T. S. Polansky and R. W. Stone,* Pennsylvania State College.

A study has been made of the microflora of cacao beans, together with some resulting chemical changes produced by the microorganisms during fermentation. Counts of viable organisms were made daily during the fermentation period. Relative percentages of types, the stage of their appearance in the fermentation, and their isolation and identification were carried out.

During the initial or alcoholic stage of the fermentation, yeast-like organisms predominate. This is followed by an acetic and later a proteolytic stage. In both these latter stages gramnegative rods are most numerous. Utilization of reducing sugars, pH, and titratable acidity were followed. Determinations were made on fermenting beans at 25°C., 36°C., and 45°C. Under the experimental conditions, variations

in temperature generally resulted in a change of rate rather than in type of fermentation.

A STUDY OF THE EPIDEMIOLOGY OF TOBACCO WILDFIRE. *H. D. Mc-Auliffe, M. A. Farrell, D. E. Haley and J. J. Reid,* The Pennsylvania Agricultural Experiment Station.

The epidemiology of tobacco wildfire has been the subject of a study carried out as a threefold program consisting of a general survey of the tobacco-growing regions of Pennsylvania, soil fertility experiments and laboratory investigations.

It has been found that the incidence of tobacco wildfire cannot be related to the incidence of *Phytomonas tabaca* inasmuch as the organism has been present in all tobacco examined, whether exhibiting lesions or free from all evidence of disease.

On the other hand, normal tobacco of the cigar-leaf filler type has been found to be very resistant to infection of economic severity during the growing season. Normal tobacco contains a nitrogen-potassium ratio of somewhat less than one. Tobacco with a ratio greater than one has been found relatively susceptible during the growing season.

During the ripening period, tobacco may become susceptible regardless of previous nutrition if any significant amount of nitrogen is taken up by the plant. This susceptibility has been found to be increased by topping and suckering, practices which take from the plant the last possibility of the normal use of nitrogen at this period in the proliferation of new tissue.

The conclusion is drawn that the control of tobacco wildfire is largely a matter of plant nutrition.

THE RELATION OF DELAYED AMMONIFICATION AND NITRIFICATION TO LEAF SPOTS OF TOBACCO. *J. Naghski, H. D. McAuliffe, D. E. Haley and J. J. Reid,* The Pennsylvania Agricultural Experiment Station.

Susceptibility of tobacco to leaf spot diseases during the ripening period has been found to be associated with a significant nitrogen uptake at this stage.

The presence of nitrates in the soil in excessive amounts during the ripening period has been shown to be due to delayed ammonification and nitrification of the organic nitrogen applied in the spring in some instances, and in others to delayed nitrification of ammonium salts which have probably remained for extensive periods in some relatively insoluble form such as ammonium magnesium phosphate.

Delay in ammonification and nitrification is usually associated with lack of sufficient rainfall in the growing season. If this condition is followed by rain during the ripening period an excessive amount of ammonification and nitrification takes place, the normal resistance of tobacco to leaf spot diseases is lost and a serious outbreak of disease occurs.

The conclusion is drawn from these studies that it is unsafe to incorporate large quantities of complex organic nitrogen in a soil to be used for tobacco. From this standpoint tobacco should not follow a legume in the rotation nor should any animal manure be applied to the crop that has not been previously rotted.

A CULTURAL AND SEROLOGICAL STUDY OF PHYTOMONAS TABACA AND CERTAIN RELATED FORMS. *J. J. Reid, J. Naghski, R. G. Harris and H. D. McAuliffe,* The Pennsylvania Agricultural Experiment Station.

A study has been made of a number of strains of *Phytomonas tabaca* and strains of a few other species of the genus *Phytomonas* associated with leaf spot diseases. These have been investigated physiologically and serologically and compared with known strains of *Pseudomonas fluorescens* and with more than 2000 isolations of *P. fluorescens* from the leaves of normal, healthy tobacco and clover.

It has been found possible through culture in unheated plant extracts high in nitrogen to bring about a physiological adaptation of *Pseudomas fluorescens* that is physiologically and serologically identical with strains of *Phytomonas tabaca*.

Such adaptation may be induced rapidly in the laboratory and may be as rapidly destroyed by frequent passage through other laboratory media.

As a result of this study the conclusion has been drawn that *Phytomonas tabaca* represents merely a transitory physiological adaptation of *Pseudomonas fluorescens*.

The work with the other representatives of *Phytomonas* associated with leaf spots while not as conclusive, suggests that other members of the genus are also temporary physiological adaptations of *Pseudomonas fluorescens*.

EASTERN MISSOURI BRANCH

NOVEMBER 8, 1938

STUDIES ON RABIES. 1. COMPARISON OF GUINEA PIG AND MOUSE INOCULATION METHODS. *S. Edward Sulkin and Joseph C. Willett*, Laboratories of the St. Louis Health Division, St. Louis, Missouri.

A simple technique for the early diagnosis of rabies by mouse inoculation is described as a convenient means of verifying the routine microscopic (Negri body) method. The results of the experiments presented show that (1) the white mouse is the animal of choice in the laboratory diagnosis of rabies, Swiss mice being of no practical advantage; (2) that the demonstration of Negri bodies in the test animal is the only dependable criterion for a positive diagnosis since clinical manifestations are frequently too variable and indefinite to be of any value; (3) that it is practical to attempt demonstration of Negri bodies by sacrificing mice on the 8th or 9th day after the inoculation; and (4) that the mouse inoculation test offers a more expedient and cheaper means of diagnosis than the guinea pig method.

STUDIES ON RABIES. 2. DEMONSTRATION OF RABIES VIRUS IN GROSSLY DECOMPOSED ANIMAL BRAINS. *S. Edward Sulkin and Nathan Nagle*, Laboratories of the St. Louis Health Division, St. Louis, Missouri.

Putrified brain tissue is usually difficult material in which to demonstrate Negri bodies and for this reason the diagnosis of a decomposed brain by microscopic examination is unreliable.

Experiments are presented describing a simple procedure for demonstrating rabies virus in grossly decomposed animal brains. In the preliminary experiments several bactericidal agents including merthiolate, glycerol, phenol, and ether were used in an effort to find one which would conveniently sterilize the heavily infected inoculum without appreciably effecting the virus. Ether used according to the method suggested by Dr. John R. Paul (J. Bact., *35*, 493,

1938) and others proved to be the most desirable agent. It was found that ether in final concentration of 10% exerts a definite bactericidal effect upon contaminated dog brains after exposure for 2 hours at 4°C. Exposure to this concentration of ether for 18 hours at 4°C. had no effect upon the virulence of two strains of street virus tested. The experiments were conducted with the view of establishing a simple and practical method for treating contaminated brains before intracerebral injection into animals.

PROPHYLAXIS OF TETANUS. *Philip Varney*, Department of Bacteriology, School of Medicine, Washington University, St. Louis.

REPORT ON THE HOT SPRINGS, ARKANSAS, CONFERENCE ON SYPHILIS. *R. B. H. Gradwohl*, Gradwohl Laboratories, St. Louis.

ERRATUM

In the article by J. Howard Mueller on "A Synthetic Medium for the Cultivation of C. Diphtheriae" in this JOURNAL for November, 1938, volume 36, the following correction should be made: In line 8 of Formula A on page 512, and in line 10 of Formula B on page 513, the figure opposite the words "complete medium without NA_2HPO_4" should, in each case, read 0.03 instead of 0.3.

THE RELATION OF AEROBIOSIS TO THE FERMENTATION OF MANNITOL BY STAPHYLOCOCCI

EUGENIA VALENTINE COLWELL

Laboratory of Industrial Hygiene Inc., New York City

While studying the fermentative reactions of a number of strains of *Staphylococcus aureus*, it was noticed that the mannitol tubes differed in appearance from those of the fermentable carbohydrates glucose, lactose, and sucrose. In the mannitol tubes (1 per cent mannitol semi-solid agar with Andrade indicator) only the top of the medium to a depth of $\frac{1}{8}$–$\frac{1}{4}$ inch was pink after 24–48 hours incubation, while in the other tubes the medium was uniformly pink. This observation led to the study of the fermentation of mannitol by staphylococci from various sources with special reference to their oxygen requirements. Six strains of mannitol-fermenting streptococci were tested in comparison.

The fermentation of mannitol by staphylococci has been studied by a number of investigators, Hine (1922), Dudgeon and Simpson (1928), Julianelle (1937), Thompson and Khorazo (1937), and others, and their conclusions are in close agreement that the pathogenic strains, (*Staphylococcus aureus* and *Staphylococcus albus*), are, in the main, mannitol-positive, while the nonpathogenic ones (usually *S. albus*) are, with few exceptions, mannitol-negative. With regard to the oxygen requirements for the growth of staphylococci, the test books list these organisms as facultative anaerobes which grow best in the presence of oxygen. Fildes and his co-workers (1936) found that a synthetic nutrient mixture that supported aerobic growth of staphylococci was inadequate for anaerobic growth without the addition of three other substances, namely pyruvic acid, CO_2, and a

"staph factor" which was shown later by Knight (1937) to consist of nicotinamide and aneurin (Vitamin B_1).

METHODS

Solutions of the test substances glucose, lactose, sucrose, and mannitol (Pfanstiehl), sterilized by Berkefeld filtration, were added in 1 per cent amounts to the basic media, 1 per cent Difco Bacto peptone, pH 7.0, and semi-solid agar containing 0.5 per cent peptone, pH 7.1. Andrade and brom-cresol-purple were used as indicators. After inoculation of a small loop of 18-hour slant growth, free access of oxygen was excluded from certain tubes by a layer of sterile vaseline on top of the medium.

SOURCE OF CULTURES

Six of the staphylococcus cultures from boils, all from normal throats, normal skin, and cream filling were freshly isolated and tested within two weeks. The rest were stock cultures isolated within five years. All colonies fished from each individual source were identical by mannitol fermentation.

Only cultures of staphylococci which were found to ferment mannitol aerobically are included in this study. Other cultures were tested which failed to ferment under both aerobic and anaerobic conditions; they included 4 *S. aureus* strains (normal throat and normal skin) and 10 *S. albus* (normal throat, normal skin and infected sebaceous gland).

The results of the fermentation tests of 25 *Staphylococcus aureus*, 5 *Staphylococcus albus*, and 6 *Streptococcus* cultures in mannitol, glucose, lactose, and sucrose mediums in test tubes with and without a layer of vaseline are summarized in the table. All of the staphylococcus cultures fermented mannitol within 48 hours when grown aerobically at 37°C. The semi-solid medium showed the indicator change only at the top of the medium during 24–48 hours incubation except for two cultures, H and C, which were slightly acid in the butt as well; by the third day of incubation all tubes were uniformly acid. When grown anaerobically, 28 of the 30 cultures were negative up to the 5th day, but after 6–14 days small and irregular amounts of indicator change were

noted. The 6 cultures of *Streptococcus* fermented mannitol within 24 hours under both aerobic and anaerobic conditions. · The carbohydrates glucose, lactose, and sucrose were fermented promptly by all of the cultures of *Staphylococcus* and *Streptococcus* when grown with and without vaseline seal.

Other basic media, namely extract broth and 10 per cent horse-serum water containing mannitol were tested with a limited

TABLE 1

Fermentation of mannitol, glucose, lactose, and sucrose by staphylococci and streptococci when tested aerobically and anaerobically (vaseline seal)

ORGANISM	SUB-GROUP	SOURCE	NUM-BER	MANNITOL			GLUCOSE, LACT., SUCROSE	
				Aerobic	Anaerobic		Aerobic	Anaerobic
				48 hours	48 hours	5 days	48 hours	48 hours
Staphylo-coccus	*Aureus*	Boils, etc.	16	+	−	−	+	+
		Boil (H)	1	+	±	+	+	+
		Blood	1	+	−	−	+	+
		Skin (normal)	2	+	−	−	+	+
		Throat (normal)	2	+	−	−	+	+
		Throat (normal) (C)	1	+	±	±	+	+
		Cream filling	2	+	−	−	+	+
	Albus	Boil	1	+	−	−	+	+
		Skin (normal)	1	+	−	−	+	+
		Throat (normal)	3	±−+	−	−	+	+
Strepto-coccus	*Alpha*	Feces	2	+	+	+	+	+
		Milk	2	+	+	+	+	+
	Beta	Milk	2	+	+	+	+	+

Explanation of symbols: +, acid; ±, weakly acid; − no change in indicator.

number of cultures. The results coincided with those obtained in the peptone and semi-solid media.

More concise data on the relation of free access of oxygen to the fermentation of mannitol by staphylococci resulted from a comparison of the pH readings on a culture incubated in a shallow layer of mannitol-peptone medium in a flask and in test tubes, with and without vaseline seal. pH readings were made by the

colorimetric method. After 24 hours the pH of the growth in the flask was 4.9, in the open tube 6.0, and in the closed tube 7.0; after 48 hours the readings of the growth in the respective environments were 4.5, 5.2, 7.0; and after 5 days 4.5, 4.5, 6.8. Four additional cultures were grown under vaseline seal in duplicate for 5 days. The pH readings ranged from 6.3 to 6.9. Duplicate tubes, in some instances, gave different pH readings, which were usually associated with slight differences in cloudiness.

GROWTH OF STAPHYLOCOCCUS UNDER ANAEROBIC CONDITIONS

The small changes in pH reading and the occasional slight increased cloudiness after 5 days incubation under vaseline seal would indicate that the organisms had grown to some extent. More definite proof was obtained when serial dilutions of culture in semi-solid mannitol-agar were incubated with and without vaseline seal. Observations made after 24 hours incubation showed that, in the highest culture dilution, approximately the same number of colonies had developed in the aerobic and anaerobic tubes, but the colonies in the closed tube were much smaller than in the aerobic one and they did not increase in size upon incubation for 5 days.

FERMENTATION OF GLYCEROL AND SORBITOL

Because of the observed difference in the fermentative reactions of staphylococci in the alcohol mannitol as compared with the carbohydrates glucose, lactose, and sucrose, it seemed of interest to test reactions in the alcohols glycerol and sorbitol. The result of this test showed that 8 mannitol-positive cultures, including strain H, fermented glycerol aerobically in 48 hours to 5 days but failed to ferment within 14 days when incubated under vaseline seal. A mannitol-negative strain of *Staphylococcus* was also negative in glycerol.

Sorbitol was not fermented by 10 cultures of *Staphylococcus* incubated aerobically for 12 days. Ten per cent horse-serum water with 1 per cent sorbitol was used in addition to the peptone and semi-solid mediums. Sorbitol was fermented within 24 hours by 2 cultures of beta-hemolytic streptococcus of Lancefield (1933) group C when grown aerobically and under vaseline seal.

DISCUSSION

The results of these experiments, which show distinct differences in the anaerobic actions of staphylococci on mannitol and glycerol on the one hand and on glucose, lactose, and sucrose on the other, but not in the aerobic actions on these same test substances, are presented without attempting to enter further into the chemical, metabolic, or enzymatic factors involved. A possible explanation of these findings may be based on difference in chemical composition, the former compounds being alcohols and the latter carbohydrates; and the probability that under anaerobic conditions the alcohols are not utilized as readily as the carbohydrates by an organism which grows feebly anaerobically. The studies of Gladstone, Fildes, and Richardson (1935) may have some bearing on this work. They found that "in an anaeroboid CO_2-free atmosphere, *Staphylococcus aureus* did not grow in 45 hours but that if glucose or lactate were present growth occurred in 24 hours."

SUMMARY

Twenty-eight cultures of *Staphylococcus* (23 *S. aureus* and 5 *S. albus*) which fermented mannitol in 24–48 hours aerobically, when tested under vaseline seal showed no change of indicator after 5 days incubation, but small and irregular amounts on longer incubation. pH readings on a limited number of cultures, incubated for 5 days under vaseline seal, ranged from 6.3 to 6.9. Two cultures of *Staphylococcus aureus* showed slight to moderate fermentation of mannitol after 48 hours incubation under vaseline seal.

Eight cultures of mannitol-fermenting staphylococci fermented glycerol aerobically in from 48 hours to 5 days, but failed to ferment when grown anaerobically for 14 days.

The fermentation of glucose, lactose, and sucrose by the 30 cultures of *Staphylococcus* took place within 48 hours aerobically and under vaseline seal.

Unlike the staphylococci, 6 *Streptococcus* cultures (4 alpha and 2 beta) fermented mannitol as well as glucose, lactose, and sucrose within 48 hours whether grown under aerobic or anaerobic conditions.

The marked inhibition or lack of fermentation of mannitol anaerobically by staphylococci is dependent, to some extent at least, upon the limited growth activity in an anaerobic environment.

It is suggested as a possible explanation that staphylococci cannot utilize the alcohols mannitol and glycerol as readily as the carbohydrates glucose, lactose, or sucrose in order to obtain substances essential for vigorous growth under anaerobic conditions.

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A SKUNK-LIKE ODOR OF BACTERIAL ORIGIN IN BUTTER[1]

T. J. CLAYDON AND B. W. HAMMER

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Butter is subject to various types of microbiologic deterioration. Some of these are encountered frequently, others quite infrequently, in the butter industry. Occasionally, a defect is noted that has not been observed previously in a given butter-producing section, or at least has not been differentiated from other objectionable conditions.

The work herein reported deals with an unusual butter defect in which the product developed a skunk-like odor. The organism responsible for the condition was isolated.

EXPERIMENTAL

General studies

Three samples of commercial butter, from a series being held under observation at 21°C. to test the keeping qualities, developed a skunk-like odor, sample 1 after 2 days and samples 2 and 3 after 4 days. The defect was most conspicuous in sample 1. The samples were unsalted and originally scored 93. They were from the same plant; another sample from the plant did not develop the defect and later samples also failed to do so.

Approximately 10-gram portions from samples 1 and 2 were inoculated separately into pasteurized sweet cream and the cream churned. The resulting lots of unsalted butter were divided and stored at 21° and about 5°C. In 2 days at 21°C. the samples developed the odor of the original butter, sample 1 causing the defect to a much greater extent than sample 2. In 6 days the

samples stored at about 5°C. developed the off odor, sample 1 again exhibiting a much more conspicuous defect than sample 2.

The procedure was repeated, using the defective experimental butter for inoculation of the cream. The defect developed in the lot representing sample 1 but not in the other. From the former sample the defect was carried through five successive churnings by inoculating cream with the defective butter made just previously; additional churnings were not attempted.

When the first experimental churnings were made the original butter was examined bacteriologically. The usual plating method was employed and also the method of Long and Hammer (1938) in which tiny portions (approximately 1/20,000 gram) are smeared on sections of agar plates. Beef-infusion agar, with skim milk and fat emulsion added just before pouring, was used. The numbers of bacteria, as shown by plating, were as follows:

Bacteria per ml.

Sample 1:		Sample 2:	
Total	31,000,000	Total	185,000,000
Proteolytic	21,000,000	Proteolytic	35,000,000
Lipolytic	9,000,000	Lipolytic ..	27,000,000

The floras of the two samples were similar and comprised three main colony types—green fluorescent, greyish white and bluish transparent. In general, the types of colonies on smeared plates were the same as on poured plates.

Pure cultures of the conspicuous colony types inoculated into pasteurized sweet cream failed to produce the skunk-like odor in unsalted butter made from the cream. Various combinations of these types, as well as cultures of less common types, also gave negative results.

The first samples of experimental butter were examined bacteriologically in the same manner as the original butter. The plate counts on the samples were as follows:

Bacteria per ml.

Sample 1:		Sample 2:	
Total	770,000,000	Total	620,000,000
Proteolytic	80,000,000	Proteolytic	110,000,000
Lipolytic	11,000,000	Lipolytic	14,000,000

In general, the colony types were the same as with the original butter. Pure cultures obtained from the defective experimental butter, as well as various combinations of these, failed to reproduce the defect.

Since the reproduction of the unusual odor in series indicated that the defect was due to an organism, attempts were made to increase the proportion of the causative type by culturing the butter in litmus milk at about 5°C. These cultures developed the skunk-like odor in 4 to 5 days. Successive transfers from them reproduced the odor in litmus milk. Unsalted butter made by inoculating pasteurized sweet cream with the cultures also developed the skunk-like odor. On streaking the litmus milk cultures, or the butter, on beef-infusion agar the same general flora developed as from the original butter.

Since the skunk-like odor suggested the breakdown of sulfur compounds, nutrient broth containing 0.02 per cent cystine was used in an attempt to increase the number of odor-producing organisms. Defective butter inoculated into this medium produced a strong hydrogen sulfide odor in 1 or 2 days. However, the same result was obtained with a sample of normal product.

Butter having the skunk-like odor was plated on beef-infusion agar containing lead acetate and cystine and colonies producing blackened areas were picked into litmus milk. The organisms thus obtained did not give the odor in experimental butter made from pasteurized sweet cream into which they had been inoculated.

At this point, it appeared that the causative organism was not developing on beef-infusion agar and additional media were employed. With each medium the conspicuous colony types were picked and tested in the usual way. One of the media consisted of 4 per cent casein and 1.5 per cent agar in water; the casein was dissolved in a small amount of water by means of sodium hydroxide and the agar in the remainder of the water by boiling, after which the two were mixed and the pH adjusted to approximately 7.0. From plates of this medium streaked with defective butter and incubated at 21°C. a number of colonies were picked that gave the skunk-like odor in litmus milk. The

colonies were rather characteristic in appearance and constituted from 5 to 10 per cent of the total number. When litmus milk cultures from these colonies were inoculated into pasteurized sweet cream and the cream churned, the resulting unsalted butter developed an odor like that of the original butter. From such butter the organism inoculated into the cream was easily recovered by smearing on the casein medium.

After the isolation of the organism from casein agar plates, beef-infusion agar plates smeared at the same time were examined for its presence. No colonies were observed on plates incubated at 21°C. but later observations showed small numbers on plates incubated at about 5°C. Subsequent trials indicated that the organism grew well on beef-infusion agar at 21°C. It was probably missed originally because of the relatively small numbers of colonies present, the percentage of the total colonies made up of the causative organism being smaller on beef-infusion agar than on casein agar as a result of the suitability of the former for growth of many species. The organism also grew on various other agar media but not on tomato agar. In some of the later trials beef-infusion agar was used for isolation.

The organism was studied in detail and was considered to be an undescribed species. The name *Pseudomonas mephitica* is suggested for it. The following description is based on a number of cultures isolated from the butter studied.

Description of Pseudomonas mephitica[2]

Morphology (at 21°C.). Form and size—Rods; in young beef-infusion agar slope cultures the cells varied from 0.5 to 1.0 by 1.5 to 14.0 microns and averaged about 0.6 by 3.8 microns. Older cultures generally had more of the longer cells.

Arrangement—Singly, pairs and chains, in preparations from agar or milk.

Motility—Actively motile with one polar flagellum which sometimes did not appear to be exactly at the end of the cell.

Staining reactions—Stained readily with the usual stains; gram-negative; granules generally present.

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[2] The description was checked by Dr. H. F. Long.

Spore formation—Spores not observed; the organism was easily destroyed by heat.

Cultural characteristics (at 21°C.). Agar slope—Growth on beef-infusion agar slopes was echinulate, greyish white and wrinkled. After 1 or 2 days the slopes had a skunk-like odor.

Agar stab—In 2 to 3 days beef-infusion agar stabs showed a greyish white, wrinkled surface growth; along the line of inoculation growth was very meager.

Agar colony—On beef-infusion agar, colonies were evident in 1 to 2 days; well developed surface colonies were convex, circular, about 3 mm. in diameter, shiny, greyish white, with an entire edge and a fine granular or feathered appearance. Older colonies were about 5 mm. in diameter, convex and slightly wrinkled at the center. Subsurface colonies were generally elliptical. All colonies were of a doughy consistency which permitted them to be picked rather completely from the agar.

Gelatin stab—Growth was essentially the same as in an agar stab and liquefaction, evident after several days, progressed slowly and was crateriform changing to stratiform.

Bouillons—Bouillons showed a turbidity, which gradually extended downward and settled as sediment, and a white, easily broken pellicle.

Dunham's solution—Growth was the same as in bouillons.

Uschinsky's solution—Slight turbidity developed and settled as a white sediment.

Potato—Growth on potato was echinulate, shiny and slightly brown.

Litmus milk—A skunk-like odor developed in 1 to 2 days, a greyish blue ring at the surface in about 3 days, and a definite alkalinity in 7 to 10 days. Reduction began at the bottom of the culture in about 7 days and was generally complete in 14 days. At this time the litmus milk was slightly proteolyzed and somewhat viscous; certain strains showed a slight viscosity earlier.

Plain milk—Except for reduction of litmus, the action in plain milk was the same as in litmus milk.

Biochemical characteristics (at 21°C.). Gas production—None.

Hydrogen sulfide—Not produced.

Indole—Not produced.

Acetylmethylcarbinol from glucose—Not produced.

Methyl-red reaction—Negative.

Nitrates—Reduced to nitrites.

Ammonia—Produced in nitrate broth and peptone broth.

Reaction change—Acid was not produced from arabinose, dextrin, galactose, glycerol, lactose, mannitol, raffinose or salicin. It was slowly produced from glucose, levulose, maltose and sucrose, with some tendency to reversal of reaction. Acid production was especially slow from glucose.

Natural fats—Not hydrolyzed.

Growth conditions. Oxygen relationships—Facultative.

Growth temperatures—Grew well at 21°C., considerably slower at 5°C., slight growth at 30°C., but none at 37°C.

Effects of various factors on development of the skunk-like odor in butter by pure cultures

In unsalted experimental butter, made by inoculating a pure culture of the organism into pasteurized sweet cream, the skunk-like odor commonly developed in 2 days at 21°C. and in about 6 days at approximately 5°C. Repeated trials gave consistent results. The effects of various factors on the development of the defect were then considered.

Effect of pH of the cream. A series of churnings was made from six portions of pasteurized sweet cream adjusted to pH values ranging from 5.6 to 7.3 at the time of inoculating the culture into the cream. After incubating overnight at about 10°C., the cream was churned. In 2 days the defect was present in all six samples of butter at 21°C. The odor was least conspicuous at the lowest pH. In another series of three portions of cream adjusted to pH values of 5.0, 6.5 and 7.5, the defect developed in all the butter at 21°C. but was somewhat less pronounced than in the first series. Again, the odor was least conspicuous at the lowest pH.

Effect of salting and working. The effect of salting and working butter on the development of the defect at 21°C. is illustrated by the following summary. The salt percentages are based on the salt added rather than on analyses of the butter.

The data, confirmed in other trials, show that the working the salted butter received was a factor in controlling the defect. While salt tended to inhibit development of the odor, it was not completely effective unless combined with thorough working.

SALT ADDED	WORKING OF BUTTER	DEFECT DEVELOPED
None............	Moderate	Pronounced after 2 days
1 per cent.......	Slight	Definite after 2 days
	Moderate	Slight after 3 days
	Thorough	Questionable after 6 days
2 per cent.......	Slight	Definite after 2 days
	Moderate	Questionable after 3 days
	Thorough	None after 6 days

Effect of contamination of the wash water.   The defect was produced in unsalted experimental butter when small amounts of pure culture were added to the water used to wash the butter. It usually was evident in 2 to 3 days at 21°C.

Relationship to other defects

Certain creameries in the southwest have reported an odor in butter which is suggestive of a skunk odor.   It is often attributed to one or more plants consumed by the cows in the pastures. Over a period of about 1 year, each of three samples of such butter was inoculated into pasteurized cream and the cream churned in order to determine whether the defect could be reproduced.   In each case the original butter was salted; the skunk odor was definite but differed somewhat from the defect produced by the culture isolated.   None of the experimental butter, which was unsalted, developed the odor of the original butter.

DISCUSSION

The development of a skunk odor in butter through the action of a bacterial species illustrates the unusual defects caused in this product by microorganisms.   The list of such defects will undoubtedly be extended as improved manufacturing procedures limit the total number of organisms in butter and still occasionally permit one or more particularly harmful species to be present in sufficient numbers to cause conspicuous changes.

The defect studied, while definitely suggesting a skunk odor, varied somewhat from one experimental lot of butter to another as a result of an increase and then a decrease in intensity.   At

certain stages the odor was comparable to that of decomposing cabbage. The similarity of the defect to an off flavor in butter that is generally regarded as due to feed is of importance in showing that organisms can produce defects suggestive of those caused in other ways.

Since *Pseudomonas mephitica* grew at relatively low temperatures, over the usual pH range used in buttermaking, and in salted butter that was only moderately worked as well as in unsalted butter, it undoubtedly would be of significance commercially when considerable numbers are added to pasteurized cream intended for butter, or to the butter itself. The low thermal resistance of the organism indicates that proper pasteurization and sanitary methods in the plant will control the defect it causes.

SUMMARY

Three samples of high quality, unsalted butter from one plant developed a skunk-like odor on holding at 21°C. to test the keeping qualities. When the defective butter was inoculated into pasteurized sweet cream and the cream churned, the defect was reproduced. The causative organism was isolated and described; the name *Pseudomonas mephitica* is suggested for it. On inoculating the pasteurized cream used for churning, the defect developed in unsalted butter made from cream having pH values from 5.0 to 7.5. Salt had an inhibitory effect on the production of the defect but thorough working of the salted butter was necessary to prevent its appearance. The skunk-like odor was produced in unsalted butter by inoculating small amounts of culture into water used to wash the butter.

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GROWTH FACTORS FOR BACTERIA

VII. NUTRIENT REQUIREMENTS OF CERTAIN BUTYL-ALCOHOL-PRODUCING BACTERIA[1]

L. E. McDANIEL, D. W. WOOLLEY AND W. H. PETERSON

Departments of Agricultural Bacteriology and Biochemistry, University of Wisconsin, Madison, Wisconsin

With the exception of *Clostridium pasteurianum* none of the butyl-alcohol-producing bacteria have been satisfactorily cultivated in synthetic media. Usually these organisms are cultured in corn mash, in yeast-water glucose, or in an inorganic salts-peptone-glucose medium (McCoy *et al.*, 1930). Apparently, there is in corn, yeast and peptone some compound or compounds essential for the growth of these organisms.

Tatum, Peterson and Fred (1935) found l-asparagine or related dicarboxylic acids necessary for a normal fermentation by *Clostridium butylicum* in corn mash.

Weizmann and Rosenfeld (1937) obtained a normal fermentation of carbohydrate by *Clostridium acetobutylicum* in a medium which included substances of known composition and a growth-promoting substance contained in a dialysate of autolyzed yeast.

Normal yields of solvents were obtained by Brown, Wood and Werkman (1938) by growing butyl-alcohol-producing bacteria in a medium of hydrolyzed casein, ammonium sulfate, tryptophane, glucose, inorganic salts and an acidic ether-soluble extract of Difco yeast extract.

This paper deals with the occurrence and partial purification of an accessory substance which permits growth of *C. butylicum* and *C. acetobutylicum* in a medium containing inorganic salts, asparagine and glucose.

Cultures and methods

The bacteria employed were *C. butylicum* strain 21 (Langlykke, Peterson and Fred, 1937), a butyl-alcohol isopropyl-alcohol-forming culture; and *C. acetobutylicum* Weizmann (McCoy, Fred, Peterson and Hastings, 1926), a butyl-alcohol acetone-producing culture. The same spore stock in soil was used throughout the study.

The basal medium contained 2 per cent glucose, 0.1 per cent asparagine, acid hydrolyzed casein, Speakman's inorganic salts,[2] reduced iron and distilled water. For *C. acetobutylicum*, 0.25 per cent hydrolyzed casein was used. Addition of tryptophane to the hydrolyzed casein did not improve fermentation. For *C. butylicum* 0.05 per cent casein was used at first, but later it was omitted and a purely synthetic base medium employed. Only slight growth occurred in the unsupplemented basal medium. The relative amounts of accessory growth factor in different source materials were determined in the early experiments by noting the comparative rates of fermentation of glucose when such materials were added to the basal medium. Each substance was tested in several concentrations, the total volume of medium in each case being 35 ml. Since a small amount of growth always occurred in the unsupplemented basal medium, each time assays were made the basal medium alone was assayed.

The inocula were prepared as follows. Each time assays were made, a fresh culture was started from the spore stock by inoculating into a medium of the same composition as that described above except that hydrolyzed casein was replaced by Bacto-Peptone (0.5 per cent for *C. acetobutylicum*, 0.25 per cent for *C. butylicum*). Each tube of medium was placed in boiling water for five minutes prior to inoculation and again for two minutes after inoculation. All cultures were incubated at 37°C. in anaerobic oat jars. Twenty-four hours later these cultures

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[2] Speakman's salts: K_2HPO_4, 0.5 gram; KH_2PO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.01 gram; $FeSO_4 \cdot 7H_2O$, 0.01 gram; and $MnSO_4 \cdot 3H_2O$, 0.01 gram per liter.
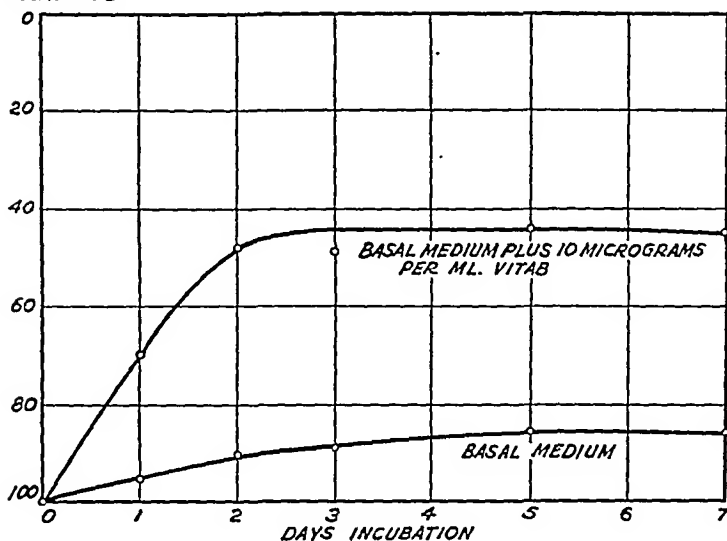
Fig. 1. Development of Turbidity in Cultures of C. butylicum
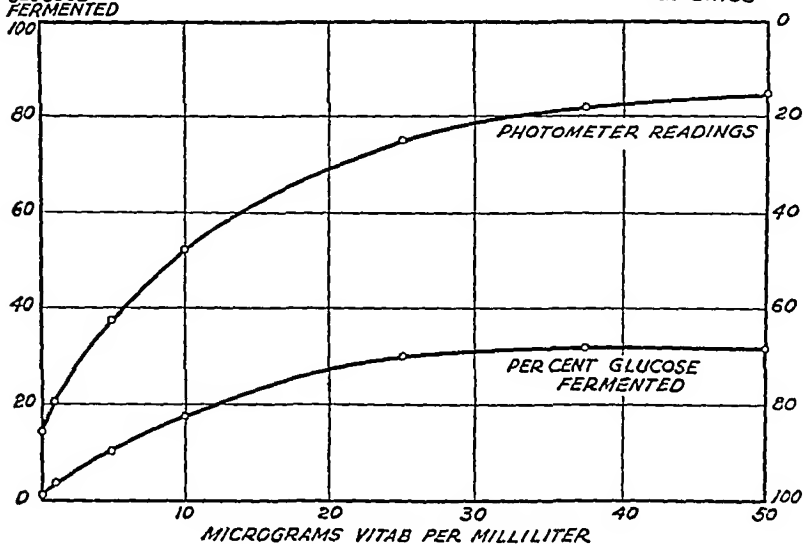
Fig. 2. Comparison of Rate of Fermentation and Culture Turbidity Assays
on Cultures of C. butylicum

were used to inoculate the assay tubes, which were also heated in boiling water for five minutes and cooled before inoculation. 0.35 ml. of inoculum was introduced into each tube. All tubes were incubated anaerobically.

Glucose fermentation was taken as an index of the amount of growth-promoting substance. Sugar was determined just after inoculation, and again after 48 hours' incubation by the method of Stiles, Peterson and Fred (1926).

In the later work, turbidity of the culture served as a criterion of growth stimulation. After three days' incubation the turbidity was determined with an Evelyn photoelectric photometer. The light intensity of the instrument was so adjusted that the uninoculated basal medium gave a scale reading of 100. The scale reading given by each culture indicated the amount of light transmitted by the culture as a per cent of that transmitted by the uninoculated basal medium. Thus, there is an inverse relation between the photometer reading and the degree of turbidity of the culture.

The maximum turbidity was reached in two to three days (fig. 1). Figure 2 gives a comparison of the two assay methods on the same cultures and shows that either method can be used for assay of the growth factor.

Sources of accessory growth factor

A growth-stimulating substance was found in many biological materials. The distribution of this substance is shown in tables 1 and 2.

Bacto-Peptone supported good growth of *C. butylicum* but rather poor growth of *C. acetobutylicum*. Hydrolyzed casein contained but little of the accessory factor for either organism. This fact suggested its use in the base medium to supply amino acids which might be essential for growth. Dried whole liver was a good source. Dialyzed yeast autolysates prepared by the method of Weizmann and Rosenfeld (1937) were stimulatory, especially for *C. acetobutylicum*. No appreciable difference was found between preparations from bakers' and from brewers' yeast. Alcoholic extracts of yellow corn were high in activity. Extractions with other solvents yielded less active preparations

and in most cases the amount of extract also was less. Alcoholic extracts of wheat bran were somewhat less active for *C. butylicum*

TABLE 1

Effects of various supplements on rate of fermentation of glucose by C. butylicum

SUPPLEMENT	ORIGINAL MATERIAL IN SUPPLEMENT	SUPPLEMENT ADDED (MICROGRAMS PER ML.) AND GLUCOSE FERMENTED (PER CENT)						
		0*	1	10	50	100	250	500
	per cent							
Bacto peptone..................	100	13.3				38.8	47.5	55.2
Hydrolyzed casein.............	100	13.3				19.6	27.6	31.1
Difco liver.....................	100	8.1				35.5	58.7	64.0
Dialysate of bakers yeast......	17.2	8.1				53.6	64.2	75.5
Dialysate of brewers yeast.....	19.7	9.0				46.2	59.5	72.1
Aqueous extract of malt sprouts.	27.8	8.1				41.7	68.5	70.2
Ethanol extract of yellow corn.	4.9	9.0		31.0	65.7	78.0	81.3	83.1
Acetone extract of yellow corn.	0.8	6.6			21.2	51.2	84.0	95.8
Butanol extract of yellow corn.	2.7	6.6		8.1	55.4	62.8	79.8	
Aqueous extract of wheat bran.	4.1	15.5	19.7	27.8		63.8		
Ethanol extract of wheat bran..	11.8	16.8		28.3	69.0	74.0		
Vitab II......................	100	10.9	12.4	33.2		46.5		
Ash of Vitab..................	6.4	3.7	2.3	3.3		4.2	1.4	0.2

* The basal medium contained 0.05 per cent hydrolyzed casein.

TABLE 2

Effects of various supplements on rate of fermentation of glucose by C. acetobutylicum

SUPPLEMENT	ORIGINAL MATERIAL IN SUPPLEMENT	SUPPLEMENT ADDED (MICROGRAMS PER ML.) AND GLUCOSE FERMENTED (PER CENT)					
		0*	1	10	100	250	500
	per cent						
Bacto peptone......................	100	19.6			20.0	22.4	23.8
Hydrolyzed casein..................	100	19.6			20.0	20.2	22.0
Difco liver.........................	100	16.3			43.8	70.9	78.4
Dialysate of bakers yeast...........	17.2	20.5			58.9	91.4	95.9
Dialysate of brewers yeast..........	19.7	21.0			43.7	79.6	95.4
Aqueous extract of malt sprouts.....	27.8	16.3			37.0	86.1	83.4
Ethanol extract of yellow corn.......	4.9	21.0		28.6	59.8	65.3	74.1
Vitab II...........................	100	19.9	22.3	29.5	51.6	60.8	

* The basal medium contained 0.25 per cent hydrolyzed casein.

than were alcoholic extracts of corn, but, because of the greater ease of extraction and higher yield, the former was preferred as a source material. The most satisfactory source found was

Vitab II,[3] a commercial product made by extraction of cereal grains. Although it was less active per unit weight than alcoholic extract of wheat bran, it has the advantage of being obtainable in large quantities in fairly concentrated form and of being completely water soluble. Alcoholic extracts of corn or bran gave turbid suspensions when taken up in water and consequently were difficult to work with.

TABLE 3

Activity of Vitab preparations for C. butylicum as indicated by turbidity of the cultures

SUPPLEMENT	ORIGINAL VITAB IN SUPPLE-MENT	AMOUNT OF SUPPLEMENT ADDED (MICROGRAMS PER ML.) AND PHO-TOMETER READINGS*			
		0†	0.1	1	10
	per cent				
Vitab II.............................	100	91.5	91.3	93.0	47.8
Neutral ether extract...................	1.9	91.5	88.0	70.0	33.8
Acidic ether extract...................	4.4	91.5	82.0	34.1	13.8
Residue from ether extraction..........	93.7	91.5	88.3	88.4	81.5
Norit filtrate........................	1.75	91.5	88.7	87.7	90.3
Norit eluate..........................	2.25	91.5	80.0	31.0	13.5
Ethyl alcohol soluble copper salts.......		90.8	37.3	20.0	15.0

* An inverse relationship exists between photometer readings and turbidity.
† Basal medium contained no casein.

Purification of the accessory growth factor for C. butylicum

The most satisfactory method for preliminary concentration of the factor was to extract a neutral aqueous solution of Vitab with ether for 70 hours to remove inactive material, and to follow this by an extraction with ether from acid solution for 100 hours to remove the growth-stimulating substance.

The active material of the acidic ether extract was completely adsorbed on norit but could be only partially regained by elution with alcohol and pyridine. While this procedure effected a certain amount of purification, a great loss in total activity occurred.

The potency of the preparation could be increased by preparing the ethyl-alcohol soluble portion of the copper salts of the concentrate. This preparation gave maximum stimulation in a concentration of about 1 microgram per ml. and was readily detectable in a concentration of 0.01 microgram per ml. Since

TABLE 4

Effect of growth-stimulating substances for other organisms upon the growth of C. butylicum

ACCESSORY SUBSTANCES	INCREASE IN GLUCOSE FERMENTATION OVER THAT OF BASE
	per cent
Base plus indole acetic acid, 0.01 microgram per ml.........	−2.0
Base plus indole acetic acid, 0.1 microgram per ml..........	1.9
Base plus indole acetic acid, 0.5 microgram per ml..........	1.7
Base plus { inositol, 50 micrograms per ml................... nicotinic acid amide, 5 micrograms per ml....... riboflavine, 0.25 microgram per ml.............. vitamin B_1, 0.5 microgram per ml............... pimelic acid, 1 microgram per ml............... amino acid mixture, 0.25 milligram per ml....... }	2.9
Base plus sporogenes growth factor, 0.1 microgram per ml...	−2.9
Base plus sporogenes growth factor, 1 microgram per ml....	−1.9
Base plus sporogenes growth factor, 10 micrograms per ml..	−0.2
Base plus vitamin B_6, 1 microgram per ml..................	−9.9
Base plus pantothenic acid, 0.1 microgram per ml...........	−0.7
Base plus pantothenic acid, 1 microgram per ml.............	1.6
Base plus β-alanine, 0.1 microgram per ml..................	−4.2
Base plus β-alanine, 1 microgram per ml...................	−2.0
Base plus β-alanine, 10 micrograms per ml.................	−5.6

only a small portion of the active material was recovered, this separation is of doubtful value.

The assays of the purified preparations are given in table 3.

At the beginning of the study, when crude materials were used as supplements, hydrolyzed casein was included in the basal medium in order to preclude the possibility of an amino-acid

deficiency. Since such small amounts of the best concentrate are required, it is improbable that any amino acid can be responsible for the stimulation. Furthermore, it does not seem possible that any amino acid could be present in the active ether extract.

TABLE 5

Growth stimulation of C. butylicum by active ether extracts after acetylation, alkali treatment, and bromination

TREATMENT	AMOUNT OF SUPPLEMENT ADDED	PHOTOMETER READINGS		
		Before treatment	After treatment	After acetylation and hydrolysis with NaOH
	micrograms per ml.			
Acetylation*............	0	64.3	52.0	52.0
	0.1	48.2	47.1	44.8
	1	17.8	31.7	28.6
	10	13.8	13.8	14.3
Steamed one-half hour in normal NaOH...........	0	91.5	91.5	
	0.1	88.0	89.5	
	1	84.0	78.0	
	10	33.0	35.0	
Bromination.............	0	85.0	85.0	
	0.1	80.0	81.0	
	1	34.8	36.0	
	10	16.1	19.7	

* In the acetylation experiments, the basal medium contained 0.05 per cent hydrolyzed casein; in the other experiments no casein was used.

Replacement of the factor for C. butylicum

A number of known growth-promoting substances were assayed in an attempt to identify the butyl factor with one of these. The data in table 4 show that indole acetic acid, sporogenes growth factor,[4] inositol, nicotinic acid amide, riboflavin, vitamin B_1, vitamin B_6,[4] pimelic acid, β-alanine, pantothenic acid,[4] and a mix-

[4] The authors wish to thank A. M. Pappenheimer, Jr., for the sample of sporogenes growth factor used, S. Lepkovsky for the Vitamin B_6, and R. J. Williams for the pantothenic acid preparation.

ture of pure amino acids failed to replace the butyl factor. The amino acid mixture contained all the naturally-occurring amino acids except hydroxy-glutamic acid. However, this amino acid could not be the active substance as it was contained in the casein hydrolysate used in the basal medium.

Stability of the factor

The growth factor for *C. butylicum* was active after heating at 120°C. for 30 minutes and after steaming for one-half hour in normal alkali. Table 5 gives evidence of its alkali stability.

Acetylation of the growth factor concentrate yielded a product which was still active for *C. butylicum*, and the activity was practically the same after hydrolysis with NaOH. This indicated, either that the active compound did not contain a hydroxyl group and could not be acetylated, or that the acetylated product was readily hydrolyzed by the organism.

Bromination of the growth factor preparation in ether solution did not perceptibly diminish its activity.

SUMMARY

Accessory growth-promoting substances for *Clostridium butylicum* and *Clostridium acetobutylicum* have been found in liver, yeast, malt sprouts, yellow corn, wheat bran, and a commercial vitamin preparation (Vitab II).

A considerable degree of purification of the stimulatory substance for *Clostridium butylicum* was accomplished by extraction with ether from acidic solution, after a preliminary extraction with ether from neutral solution to remove inactive substances. The active material was completely adsorbed on norit but could not be completely eluted with alcohol and pyridine. Alcohol-soluble copper salts gave detectable stimulation at a concentration of 0.01 microgram per ml. and maximum effect at 1 microgram per ml.

The butyl factor can not be replaced by riboflavin, indole acetic acid, sporogenes growth factor, nicotinic acid amide, vitamin B_1, vitamin B_6, pimelic acid, inositol, β-alanine, pantothenic acid, or by a mixture of all the naturally occurring amino acids except hydroxy-glutamic acid.

The factor is stable to autoclaving, to steaming in normal alkali and to bromination.

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EFFECTS OF SULFANILAMIDE ON BRUCELLA MELITENSIS, VAR. MELITENSIS, ABORTUS, AND SUIS

E. E. MENEFEE, Jr.[1] AND MARY A. POSTON

Departments of Bacteriology and Medicine, Duke University School of Medicine, Durham, N. C.

Sulfanilamide is a valuable therapeutic agent in certain bacterial infections but is not a universal panacea, since it is of little or no benefit in some diseases. Favorable reports on the action of sulfanilamide in Brucellosis have been published in the European literature by Thevenet, Grous, Berger, Suchier, Richardson, and Francis. More recent articles by Toone and Stern have appeared in the American literature, citing cases of undulant fever apparently cured by this drug.

This work was undertaken in an attempt to determine the effect of sulfanilamide on *Brucella* organisms *in vitro* and on experimental infections in a susceptible animal. The problem was divided into four parts: (1) the *in vitro* action of sulfanilamide on *Brucella* organisms; (2) the *in vitro* action of related compounds;[2] (3) the *in vitro* action of sulfanilamide and antibrucella serum, and (4) the action of sulfanilamide on experimental Brucellosis in the guinea pig.

1. In vitro action of sulfanilamide on Brucella organisms. A one-tenth-per-cent solution of sulfanilamide (p-Aminobenzenesulfonamide) was made in beef-infusion broth, pH 7.3, sterilized by passing through a Seitz filter, and the concentration of 100 mgm. per cent checked by chemical determination.[3] Dilutions

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[1] James A. Greene Research Fellow for the Study of Brucellosis.

[2] We are indebted to Mr. R. O. Roblin, Jr., of the American Cyanamid Co. for furnishing us with these related compounds.

[3] All chemical determinations were done by Dr. Ann Yates, of the Department of Biochemistry, Duke University.

of the filtrate were made up to 10 cc. with the same stock broth
so that the final concentrations of the drug were 5, 10, 20, 30,
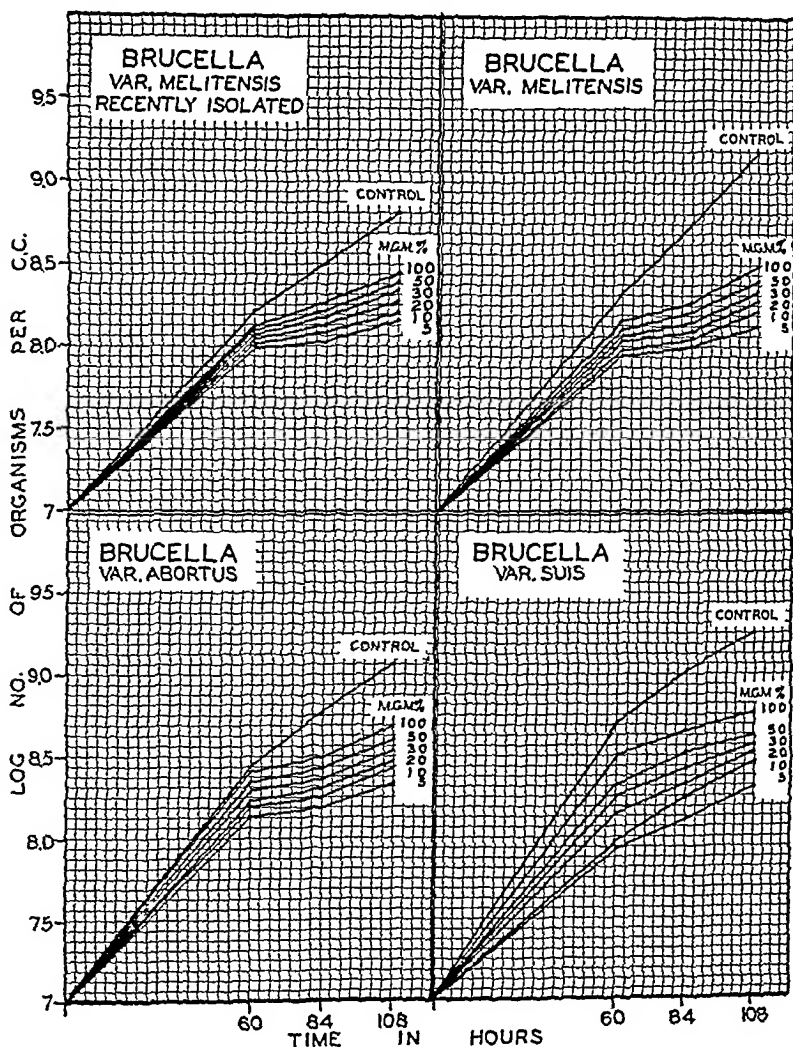50, and 100 mgm. per cent respectively.   Forty culture tubes

FIG. 1. GROWTH CURVES OF 3 VARIETIES OF BRUCELLA (4 STRAINS) IN DIFFERENT
CONCENTRATIONS OF SULFANILAMIDE

of each concentration were inoculated by adding a suspension
of 100,000,000 *Brucella* organisms (0.1 cc. of a suspension con-
taining 1,000,000,000 organisms per cubic centimeter).   Read-

ings on the Photronreflectometer (Libby) were taken after 60, 84, and 108 hours incubation at 37°C. Smears from each tube were examined after the final readings to exclude contamination, and chemical determinations showed the concentration of sulfanilamide to be unchanged and unconjugated. The pH of the media also remained unchanged (pH 7.3).

TABLE 1

Brucella organisms

HOURS	CONTROL	100 MGM. PER CENT	50 MGM. PER CENT	30 MGM. PER CENT	20 MGM. PER CENT	10 MGM. PER CENT	5 MGM. PER CENT
Variety Melitensis (428)							
60	220*	102	110	122	128	132	144
84	540	122	140	148	160	190	220
108	1180	160	190	238	270	310	390
Variety Abortus (456)							
60	258	122	136	168	175	195	210
84	520	132	164	196	212	240	320
108	1070	175	238	304	390	440	505
Variety Suis (obtained from Dr. Huddleson)							
60	490	90	130	160	172	200	320
84	1100	130	200	308	420	506	710
108	1908	210	306	412	538	644	920
Variety Melitensis (recently isolated from human)							
60	162	104	112	118	125	134	140
84	300	110	122	138	150	158	190
108	660	140	165	200	230	270	300

* Figures represent organisms in millions per cubic centimeter as determined from the average growth of forty tube cultures.

Figure 1 shows the growth curves illustrating the bacteriostatic action of various dilutions of sulfanilamide (average of 40 tubes) on 3 varieties (4 strains) of *Brucella*. Readings taken on several occasions, after 36 hours of incubation, showed no essential differences between the cultures with sulfanilamide and the controls. After 36 hours, however, the bacteriostatic effect of the sulfanilamide was very apparent (table 1). No observations on growth after 108 hours were made. Several cultures of

broth, containing 200, 300, 400, 600, and 800 mgm. per cent of sulfanilamide respectively, showed no significantly greater bacteriostatic action than the broth containing 100 mgm. per cent, and the cultures were not sterilized.

These results are similar to those obtained by Long, who showed the *in vitro* bacteriostatic effects of sulfanilamide on streptococci and other organisms. We did not confirm the work of Francis who was able to sterilize cultures of a strain of *Brucella*, var. *abortus*, by the addition of small amounts of sulfanilamide. The good results obtained by him may have been due to the small inoculum used in his experiments.

TABLE 2

Growth of Brucella, var. melitensis, after 84 hours incubation with varying amounts of sulfanilamide

DRUG	5 MGM. PER CENT	10 MGM. PER CENT	CONTROL
Sulfanilamide	200*	140	Average of 10
p,p′ Dihydroxydiphenyl Sulfide	0	0	tubes—350
p,p′ Diaminodiphenyl Sulfoxide	300	225	
S-1	200	150	
L-167	210	145	
Disulfanilamide .	190	135	

* *Figures refer to millions of organisms per cubic centimeter.*

2. In vitro action of related compounds on Brucella. var. melitensis. Because of the relative insolubility of the related compounds, concentrations of 5 and 10 mgm. per cent only were used. The technique was the same as described under experiment 1, and the results (table 2) indicate that the compounds tested except one (p,p′ Diaminodiphenyl Sulfoxide) had no greater *in vitro* bacteriostatic action than sulfanilamide.

.3. Effect of specific serum on bacteriostatic effect of sulfanilamide. Osgood showed that sulfanilamide combined with specific immune sera has a greater anti-bacterial action than either alone. Rabbit serum with a high agglutinating titre (1–50,000), a strong opsonocytophagic reaction (Evans' 'technique), but weak bactericidal properties was diluted 1:10, 1:100,

and 1:1000 in beef-infusion broth pH 7.3, containing 10 mgm. per cent sulfanilamide. After sterilization by filtration, 40 tubes containing 10 cc. of each dilution, and controls of beef-infusion broth and beef-infusion broth plus 10 mgm. per cent sulfanilamide without serum, were inoculated with *Brucella*, var. *melitensis*. Bacterial counts were made after 60, 84, and 108 hours of incubation at 37°C., and the results expressed in table 3 show that the combination of this serum with sulfanilamide was less efficient than sulfanilamide alone.

4. Effect of sulfanilamide on experimental Brucellosis in the guinea pig. Forty healthy guinea pigs were injected intraperitoneally with 1,000,000,000 organisms of a culture of *Brucella*, var. *melitensis*, recently isolated from the blood of an acutely

TABLE 3

Brucella, var. melitensis 10 mgm. per cent sulfanilamide plus serum

HOURS	CONTROL	NO SERUM	SERUM 1:10	SERUM 1:100	SERUM 1:1000
60	200*	140	180	160	150
84	320	160	300	220	200
108	710	300	700	540	410

* Figures refer to millions of organisms per cubic centimeter.

ill patient. Five days after injection, one-half of the animals were fed sulfanilamide for a 10 day period, receiving 60 mgm. per kilo per day. The dose was then halved and continued for 10 more days. At intervals (table 4) the animals were skin tested and their blood examined for sulfanilamide concentration, agglutinin titre, and opsonocytophagic reaction. Thirty-five days after inoculation all animals were killed, carefully necropsied, and the spleens cultured on liver infusion agar. All the guinea pigs developed agglutinins and opsonocytophagic powers (tables 4 and 5), but at the end of the experimental period the average agglutinin titre of the treated group had fallen and the opsonocytophagic reaction had increased. The agglutinin titre of the untreated group remained high till the end of the experiment, and the opsonocytophagic reactions were only moderately strong. *Brucella* was isolated from 19 of the 20 untreated guinea

TABLE 4

Sulfanilamide treated guinea pigs

Agglutinin titre, opsonocytophagic, skin test, and blood concentration of sulfanilamide in 20 guinea pigs infected with *Brucella*

GP	15 DAYS AFTER INJECTION				25 DAYS AFTER INJECTION				35 DAYS AFTER INJECTION				SPLEEN CULTURE
	Agglutinins	Opsonocytophagic	Skin test	Blood sulf.	Agglutinins	Opsonocytophagic	Skin test	Blood sulf.	Agglutinins	Opsonocytophagic	Skin test	Blood sulf.	
				mgm. per cent				mgm. per cent					
1	1:160	Weak	0	18	1:160	Moderate	0	10	1:20	Strong	0	0	0
2	1:320	Weak	0	13	1:320	Very strong	0	8	1:40	Very strong	+	0	0
3	1:80	Moderate	0	14	1:160	Strong	0	7	1:20	Strong	+	0	0
4	1:80	Weak	0	19	1:80	Moderate	0	11	1:40	Strong	0	0	0
5	1:160	Weak	0	23	1:80	Strong	0	17	1:20	Very strong	+	0	0
6	1:80	Weak	0	12	1:80	Strong	0	4	1:20	Strong	0	0	0
7	1:160	Weak	0	18	1:160	Moderate	0`	6	1:20	Strong	0	0	0
8	1:320	Moderate	0	16	1:320	Strong	0	6	1:20	Strong	0	0	0
9	1:160	Moderate	0	14	1:320	Strong	0	5	1:40	Very strong	0	0	0
10	1:320	Weak	0	13	1:160	Moderate	0	7	1:20	Very strong	0	0	0
11	1:20	Weak	0	11	1:20	Weak	0	7	1:20	Weak	+	0	+
12	1:320	Moderate	0	19	?1:320	Strong	0	9	1:80	Strong	0	0	0
13	1:80	Weak	0	17	1:160	Moderate	0	8	1:20	Strong	+	0	0
14	1:160	Moderate	0	16	1:320	Moderate	0	11	0	Strong	0	0	0
15	1:160	Moderate	0	12	1:80	Very strong	0	10	0	Very strong	0	0	0
16	1:80	Weak	0	18	1:40	Moderate	0	6	1:20	Very strong	+	0	0
17	1:320	Moderate	0	20	1:640	Strong	0	8	1:80	Very strong	+	0	0
18	1:160	Weak	0	14	1:320	Very strong	?	6	0	Strong	0	0	0
19	1:160	Weak	0	9	1:80	Moderate	0	3	1:40	Very strong	0	0	0
20	1:320	Moderate	0	13	1:320	Strong	0	4	1:640	Weak	+	0	0

pigs and from only 1 of the 20 treated animals. The guinea pig in the latter group which had a positive culture never developed any marked serologic reaction.

TABLE 5

Agglutinin titre, opsonocytophagic, and skin test in 20 guinea pigs infected with Brucella

GP	15 DAYS AFTER INJECTION			25 DAYS AFTER INJECTION			35 DAYS AFTER INJECTION			SPLEEN CULTURE
	Agglutinins	Opsonocytophagic	Skin test	Agglutinins	Opsonocytophagic	Skin test	Agglutinins	Opsonocytophagic	Skin test	
21	1:320	Weak	0	1:1280	Moderate	+	1:640	Strong	++	0
22	1:180	Weak	0	1:320	Weak	0	1:640	Weak	0	+
23	1:80	Weak	0	1:640	Weak	+	1:1280	Moderate	+++	+
24	1:80	Weak	0	1:160	Weak	+	1:320	Moderate	++	+
25	1:80	Weak	0	1:160	Weak	+	1:640	Weak	++	+
26	1:80	Weak	0	1:320	Weak	0	1:320	Weak	0	+
27	1:320	Weak	0	1:320	Weak	+	1:640	Moderate	+++	+
28	1:160	Weak	0	1:160	Weak	0	1:320	Weak	0	+
29	1:40	Weak	0	1:80	Moderate	0	1:640	Moderate	+	+
30	1:160	Weak	0	1:320	Weak	0	1:640	Moderate	+	+
31	1:320	Weak	0	1:160	Weak	0	1:160	Weak	0	+
32	1:40	Weak	0	1:40	Weak	+	1:320	Weak	++	+
33	1:20	Weak	0	1:80	Weak	0	1:160	Weak	0	+
34	1:320	Negative	0	1:320	Weak	0	1:320	Weak	0	+
35	1:160	Weak	0	1:80	Weak	0	1:640	Moderate	++	+
36	1:320	Weak	0	1:640	Moderate	+	1:1280	Weak	+	+
37	1:40	Negative	0	1:320	Weak	+	1:640	Moderate	+++	+
38	1:80	Weak	0	1:640	Weak	0	1:1280	Moderate	0	+
39	1:160	Weak	0	1:320	Weak	0	1:640	Weak	0	+
40	1:160	Weak	0	1:320	Weak	0	1:320	Weak	+	+

DISCUSSION

In reviewing the literature, one is impressed by the fact that in the cases of Brucellosis reported to date, every patient who responded well to sulfanilamide therapy had a high agglutinin titre before the chemo-therapy was begun. Bethoux reported a patient with *Brucella* bacteremia who had a weak agglutinin titre, and he was unable to sterilize the circulating blood by sulfanilamide therapy. Our findings in guinea pigs support the clinical observations in suggesting that the bacteriostatic action

allows the normal defense mechanisms of the body to cope adequately with the invading bacteria.

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CULTURAL REQUIREMENTS FOR THE PRODUCTION OF BLACK PIGMENTS BY BACILLI

FRANCIS E. CLARK AND NATHAN R. SMITH

Soil Microbiology Investigations, Bureau of Plant Industry, United States Department of Agriculture

Spore-bearing aerobic bacteria[1] capable of producing striking black pigments have been known since the early reports of Gorini (1896) on *"Bacillus lactis-niger"* and of Biel (1896) and Lunt (1896) on *"Bacillus mesentericus-niger."* Although these types have become established in the bacteriological literature under the respective binomial designations of *Bacillus niger* (Migula, 1900) and *Bacillus aterrimus* (Lehmann and Neumann, 1896), there has been little agreement upon their characterization or upon the cultural conditions necessary for their pigmentation.

Ford (1927) noted that *B. aterrimus* blackened potato very strikingly, in contrast to the brown color produced by *B. niger.* On the other hand, the differential key in Bergey's Manual (1934) employed the blackening of potato in the reverse order for separating the two species—*B. aterrimus*, white to pink; *B. niger*, black. Levine and Soppeland (1926) found both species capable of blackening potato, but only *B. niger* capable of fermenting lactose. This character was therefore used to separate the two species. Lehmann, Neumann and Breed (1931) considered the pigment-producing abilities of these organisms to be variable or inconstant, and grouped them together with forms closely related to *Bacillus vulgatus.*

Other species capable of producing a black coloration have been described. Carbone and associates (1921) refer to *Bacillus tyrosinogenes* of Rusconi; Fabian and Nienhuis (1934) found *Bacillus nigrificans* in spoiled pickles; and Cameron, Esty and Williams (1936) described *Bacillus betanigrificans* capable of blackening beets and culture media in the presence of iron. The relationships of these proposed species to *B. niger* and *B. aterrimus* have not hitherto been defined.

CULTURES STUDIED

Observations reported in this paper were made upon a collection of 40 cultures, 12 of which were received as named species, 7 were received unnamed but known to produce pigment, and 21 were isolated by the authors. These cultures are listed below under the three species into which they naturally fall because of their physiological characteristics. The name under which the culture was received and the history as far as known, is given opposite the number of the culture as it exists in our collection.

I. *Bacillus niger*

220   *B. mesentericus*, var. *niger*, from AMNH (733) in 1923; Kral.

229   Black bacillus, Smith, isolated in 1912.

254   *B. lactis-niger*, from Gorini (2) in 1936.

264   *B. niger*, from Hall (799) in 1936; Ford.

265   Black bacillus, from Hall (1509) in 1936.

650   *B. niger*, from Cameron in 1937; Breed; NCTC 2736; Gorini.

651   *B. niger*, from Cameron in 1937; Breed; NCTC 2592; Ford (6).

655   *B. lactis-niger*, from Cameron in 1937; Breed; Gorini (2).
       18 isolates from soil and air, 1936.

II. *Bacillus aterrimus*

230   Black bacillus, from Thom in 1936 (isolated in 1912).

259   Black bacillus, from Hall (581) in 1936.

260   Black bacillus, from Hall (620) in 1936.

261   Black bacillus, from Hall (621A) in 1936.

262   Black bacillus, from Hall (622) in 1936.

353   *B. tyrosinogenes* (Rusconi), from Hall in 1937; Istituto Sieroter., Milan.

624   *B. nigrificans*, from Porter in 1937; Fabian.

653 *B. aterrimus*, from Cameron in 1937; Breed; NCTC 2591; Ford
     (5B)
659 *B. nigrificans*, from Cameron in 1937; Fabian.
     3 isolates from soil, 1936.

III. *Bacillus betanigrificans*

648 *B. betanigrificans*, from Porter in 1937.
649 *B. betanigrificans*, from Cameron in 1937.

In addition to these cultures, 6 cultures were received as *B. aterrimus* and 6 as *B. niger* (or *B. lactis-niger*), which could not be identified as belonging to either of these species.

INFLUENCE OF THE MEDIUM ON PIGMENTATION BY B. NIGER

The importance of the substrate in the development of pigment by *B. niger* was evident when cultures, previously coal-black, failed to produce pigment when transferred to another lot of nutrient agar. These cultures, however, readily produced pigment when returned to the original medium. To overcome the supposed deficiency in the one medium, various modifications, such as the addition of carbohydrates or mineral salts, singly and in combination, or the changing of the pH value, were made without success. Finally, all brands of commercial peptones available in this laboratory were used in agar. On 5 peptone agars, *B. niger* produced blackening (see table 1) but failed to do so on the remaining 4; *B. aterrimus* failed on all. The addition of beef-extract paste to 2 of the 4 peptone agars not blackened by *B. niger* resulted in blackening; the growth of *B. niger* upon beef-extract agar alone, however, was colorless.

The addition of glucose or maltose inhibited the production of pigment on those peptone agars which were normally blackened by *B. niger*. This suggested that certain break-down products of protein were necessary for the blackening and that fermentable carbohydrates spared the protein from decomposition. Since the various commercial peptones are known to vary in their amino-acid content as well as in other respects, correction of those peptones unsuitable for pigmentation was attempted. Glycine, alanine, aspartic acid, leucine and tyrosine were added singly to

nutrient agar prepared from peptone "A"[2] B. niger produced a black pigmentation only on the nutrient agar to which tyrosine had been added. The addition of glucose or maltose to this medium did not interfere with the development of the pigmentation as it did in the cases mentioned above.

In addition to those media already mentioned, milk agar (equal volumes of skim milk and 4 per cent agar-agar) is suitable for B. niger, the black pigment usually appearing in a few days.

TABLE 1

Pigmentation responses of B. niger and B. aterrimus on various peptone agars

PEPTONE	1.0% PEPTONE		0.5% PEPTONE 0.3% BEEF EXTRACT		0.5% PEPTONE 0.3% BEEF EXTRACT 1.0% GLUCOSE	
	B. niger	*B. aterrimus*	*B. niger*	*B. aterrimus*	*B. niger*	*B. aterrimus*
A	−*†	−‡	−†	−‡	−†	+
B	+	−	+	−	−	+
C	+	−	+	−	−	+
D	+	−	+	−	−	+
E	+	−	+	−	−	+
F	−	−	+	−	−	+
G	−	−	−	−	−	+
H	+	−	+	−	−	+
I	−	−	+	−	−	+

* + black pigment present; − black pigment absent.
† Blackening with free tyrosine added.
‡ No blackening with free tyrosine added.

RÔLE OF FERMENTABLE CARBOHYDRATES IN THE PIGMENTATION OF
B. ATERRIMUS

The reports of many previous workers that carbohydrate compounds are especially desirable for the black pigmentation of *B. aterrimus* have been confirmed. *B. aterrimus* cultures failed to show blackening on any of the peptone, peptone-beef, or tyrosine-peptone-beef agars blackened by *B. niger*. However, the addi-

[2] None of our cultures of *B. niger* blackened nutrient agar containing this peptone (Difco's Bacto-Peptone). Of the 9 peptones studied, nutrient agars prepared with Difco Bacto-Tryptone or Bacto-Proteose Peptone were the most readily blackened by *B. niger*.

tion of a fermentable carbohydrate to any type of peptone agar that would support at least a moderate growth of *B. aterrimus* rendered that medium favorable for pigmentation by this species, regardless of whether or not the peptone was a type blackened by *B. niger*. The differences in the types of media which permit pigmentation of *B. aterrimus* and *B. niger* are shown in table 1.

Broquin-Lacombe (1913) recognized that *B. aterrimus* had the ability to blacken an inorganic nitrogen medium containing a fermentable sugar, whereas *B. niger* lacks this ability. We have noted blue or black pigments in *B. aterrimus* cultures upon monobasic ammonium phosphate agar with such carbon sources as xylose, arabinose, glucose, levulose, maltose, sucrose, starch, dextrin, salicin, or mannitol.

All cultures of *B. aterrimus* and the majority of cultures of *B. niger* blackened sterilized potato. In view of the differences obtained on other media, it is probable that *B. aterrimus* produces pigment on potato by means of a different enzyme system than does *B. niger*. Muschel (1922) expressed the opinion that *B. aterrimus* produces a polyphenyloxydase blackening of carbohydrate condensation products. It is probable that *B. niger* produces a melanin-type pigment by means of a tyrosinase system. For either system, potato, or peptone agar containing both fermentable sugar and free tyrosine, serve as satisfactory substrata for pigment production.

Although we are in agreement with Levine and Soppeland (1926) that either species may produce blackening on potato, we do not agree that lactose fermentation by *B. niger* suffices to separate it from *B. aterrimus*. For this determination the synthetic carbohydrate agars with brom-cresol-purple indicator were used as recommended in the Manual of Methods for Pure Culture Study, edited by the Committee on Bacteriological Technique of the Society of American Bacteriologists. Growth and more or less acid formation within 2 weeks indicated a positive reaction, whereas no growth or a very scant growth and no acid indicated a negative reaction. All of our isolations of *B. niger* are lactose negative, as was the *B. niger* (culture 254) received directly from Gorini in 1936. On the other hand, cultures of *B. niger* from the

American Type Culture Collection (culture 220) and from Hall
(culture 264) were lactose positive. All cultures of *B. aterrimus*
were lactose negative. Therefore, lactose fermentation does not
appear an adequate differential criterion. It has been previously
reported (Clark and Smith, 1938) that several species of aerobic
spore-formers regularly produce slow or mutant fermentations on
certain sugars. For instance, *Bacillus megatherium* was noted to
vary on mannose, *Bacillus cereus* and *Bacillus mycoides*, on
sucrose, and *Bacillus vulgatus*, on lactose.

The cultures of *B. nigrificans* and of *B. tyrosinogenes* (Rusconi)
were identical in every respect to type cultures of *B. aterrimus*;
they produce similar blackening on sugar media, and are con-
sidered to be synonymous.

CULTURES DISTINCT FROM B. NIGER AND B. ATERRIMUS

B. betanigrificans exhibited morphologic and cultural differ-
ences, especially the production of gas on carbohydrate media
which readily separates it both from *B. niger* and *B. aterrimus*.
It was not found to produce a black pigment on potato, milk agar,
or ordinary peptone or sugar agars, but it does produce a brownish
or black pigment in the presence of metallic iron in various media,
as originally reported by Cameron, Esty and Williams (1936).

As stated in the forepart of this paper, a total of 12 cultures
received as *B. aterrimus* or *B. niger* were identified as species
other than either of these. The donor of certain cultures stated
that he had never seen any pigmentation with those particular
cultures, but that they undoubtedly did produce color when they
were isolated. This apparent variability in pigment production
has led some bacteriologists[3] to believe that this character is
transient, whereas others believe it a stable character, although
at the moment lacking proof for their opinion except as they have
observed their own cultures. Of these 12 mislabelled cultures,
only 3 were identified as *Bacillus vulgatus*, a closely allied species,
whereas 9 were distinctly different in their reactions.

Cultural similarities between *B. vulgatus*, *B. niger* and *B.*

[3] Personal communications.

aterrimus made it desirable to determine whether pigment production was a stable character and whether variants might be developed. Consequently, cultures of *B. vulgatus* were subcultured repeatedly in enrichment broths without any pigmented strains being obtained. Attempts were also made to produce non-pigmented strains from cultures of *B. niger* and *B. aterrimus*. Cultures were aged in acid, neutral or alkaline broths (pH 5.5, 7.0, 8.0) and plated out at varying intervals. Smooth and rough colony forms were picked from these platings for further aging and plating. In no case was it found possible to obtain a non-pigmented daughter strain from an originally pigmented parent culture. Finally, it may be stated that *B. niger* (number 229) and *B. aterrimus* (number 230) have been maintained in this laboratory for 26 years without any special care and without loss of their characteristic type of pigmentation.

SUMMARY

Bacillus niger produces a black pigment upon protein media which contain free or metabolically available tyrosine. Some commercial peptones containing no readily available tyrosine are not blackened unless free tyrosine is added. The addition of a fermentable sugar to many protein media normally blackened by *Bacillus niger* inhibits pigmentation unless free tyrosine is added.

Bacillus aterrimus blackens media containing fermentable carbohydrates, either in the presence or absence of tyrosine, but does not blacken sugar-free peptone media which are readily blackened by *Bacillus niger*. Carbohydrate media containing mineral nitrogen are also blackened by *Bacillus aterrimus* but not by *Bacillus niger*.

Cultural requirements for the production of pigment by *Bacillus aterrimus* and *Bacillus niger* are believed sufficient to warrant their recognition as separate species. Since non-pigmented variants could not be developed and since a culture of each has been maintained for 26 years without loss of pigmentation, it seems that this is a stable character.

Bacillus nigrificans (Fabian) and *Bacillus tyrosinogenes* (Rus-

coni) were found similar in all respects to *Bacillus aterrimus*, and are believed to be synonymous with that species.

Bacillus betanigrificans produces black coloration in the presence of metallic iron, but not upon the media readily blackened by the species considered above. It possesses morphological and cultural properties that easily separate it from *Bacillus niger* and *Bacillus aterrimus*.

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FACTORS LIMITING BACTERIAL GROWTH

IV. The Age of the Parent Culture and the Rate of Growth of Transplants of Escherichia Coli

A. D. HERSHEY

Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Missouri

Contrary to the earlier idea that the initial lag in the multiplication of bacteria represents a period of latency, it is now recognized that actually this is a period of intense growth during which the cells are increasing in size and metabolic activity (Huntington and Winslow, 1937). Nevertheless, the new facts have been regarded as manifestations of rejuvenescence, and the description of lag as an interval of preparation for active multiplication has been retained (Topley and Wilson, 1936). Obviously, if it is to conform to the usual definitions, rejuvenescence of bacterial cells must be marked by an increased capacity for growth and metabolism which is independent of the increase in size. We have already shown to our satisfaction that when environmental conditions are held constant, the age of the source culture of *Escherichia coli* does not influence the rate of oxygen consumption per unit of bacterial substance (Hershey and Bronfenbrenner, 1938). At the same time indirect evidence was obtained that the initial rate of growth of cells transplanted from young and old cultures is the same, and this was substantially confirmed by the observation that under favorable conditions growth in broth regularly occurs without lag, if changes in the size of cells are taken into account (Hershey, 1938). The experiments to be reported at the present time are concerned with the direct measurement of the rate of increase in bulk of bacterial substance in relation to the age of the culture of *E. coli*.

The few data of this kind contained in the literature do not

bear directly on the question of age. However, certain observations on the rate of growth during the phase of lag should be referred to. Henrici (1928) constructed curves representing the growth in total cell length of microcolonies of *Bacillus megatherium*, and noted a marked shortening of the lag period as compared with curves representing the uncorrected cell count. A similar peculiarity was found by Alper and Sterne (1933) in curves obtained by photo-electric measurement of the opacity of cultures of *Salmonella gallinarum*.

Adolph and Bayne-Jones (1932) and Bayne-Jones and Adolph (1933) computed rates of volume increase of individual cells from motion picture photomicrographs of agar cultures of *B. megatherium* and *E. coli*. Occasional cells initiated growth at the maximal rate, but lag was noticeable in most cases. The considerable differences in behavior of individual cells were not clearly correlated with the age of the parent culture.

Coombs and Stephenson (1926) have described a gravimetric method for determination of bacteria, but this appears not to be applicable to measurement of initial rates of growth. A similar method has been used by Bach (1937), however, in following the development of *Staphylococcus aureus*. This author obtained curves of increase in bacterial mass which he believed to be atypical, a conclusion evidently proceeding from misinterpretation of data. No observations of the initial growth period were made.

The observation of lag has thus been the rule whatever the method of measuring rate of growth, and the inference has usually been made that the early increase in rate is a reflection of intrinsic changes in cell physiology. Our previous experiments with *E. coli* (*loc. cit.*) have failed throughout to confirm these suggestions, and the results reported below seem to give decisive support to our earlier evidence that the age (i.e., phase of growth) of cells is not one of the factors influencing the rate of growth of this organism. Lag, when it is observed, we are inclined to attribute to initially unfavorable conditions of growth quite distinct from any peculiarity inherent in the cells.

In the following experiments we have applied three different

methods to the measurement of growth in cultures of *E. coli*, each of which has proved to yield authentic values for total increase of bacterial substance.   Since these methods were suited to the study of cultures in liquid media, the findings could be directly correlated with existing knowledge of bacterial multiplication.   And since the rates obtained were averages exclusive of individual variations, they could be conveniently related to the age of the parent culture.

Considerable care has been exercised to obtain physiologically "young" and "old" bacteria of constant properties.   The strain of *E. coli* used was a stock culture subjected to repeated plating and dried *in vacuo* in sealed ampoules by the method of Brown (1926).   To insure against dissociation during the course of the work, a new transplant was made each week from the same lot of desiccated culture and maintained in the interval by daily transfer in broth.   A single lot of medium, consisting of a stock solution of 10 per cent Bactopeptone, 5 per cent Difco beef extract, and $M/10$ phosphate buffer of the desired pH, filtered and autoclaved, and diluted to 10 volumes with sterile 0.5 per cent saline as needed, was used throughout each series of measurements.   Seeded in 200 ml. amounts in one-liter Erlenmeyer flasks with 0.02 ml. (2×10^7 bacteria) of a similar culture 24 hours old, cultures yielding reproducible growth curves were obtained.   For the experiments with young bacteria, cultures prepared in this manner were centrifuged approximately three hours after seeding, at which time the organisms have attained maximal size and are just entering the phase of logarithmic multiplication.   For old bacteria, cultures of 24 hours or more were used.[1]   Accompanying each experiment, counts of viable cells have been made to verify that the rate of multiplication corresponded with that of young or old bacteria respectively, and to compute the average size of cells.   In addition a few trials were made to determine whether the organisms in the three-hour cultures would show the increased susceptibility to dele-

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[1] In these, and similar unreported experiments, no significant change in growth capacity or metabolic activity has been observed in the viable cells remaining in cultures from one to seven days old.

terious influences commonly ascribed to "physiologically young" cultures (Sherman and Albus, 1923). In every case these various evidences fully justified our choice of conditions for obtaining young and old bacteria. (See tables 1, 3 and 4.)

THE ASSAY OF BACTERIAL SUBSTANCE

Total bacterial substance was estimated for measurement of growth rate by means of a photo-electric nephelometer suited to quantitative measurements (Stier, Arnold, and Stannard, 1933–4), by determination of centrifugable nitrogen (Mueller, 1935), or by a manometric method.

The first of these was found to be the most convenient, and, when used with the proper precautions, extremely accurate.

TABLE 1

Survival of young and old bacteria in distilled water

AGE OF CULTURE	DILUTION	DILUTING FLUID	NUMBERS PER ML.* AFTER			
			0 minutes	15 minutes	30 minutes	60 minutes
hours						
24	10^{-7}	Distilled water	109	113	85	55
3	10^{-4}	Distilled water	90	1	0	0
3	10^{-4}	Saline	88	90	87	79

* Average of three plates.

The recommendations of the author regarding the method of expressing optical density, and of calibration, were followed in detail. The excellent discussion of this instrument by Longsworth (1936) was also found helpful. It was convenient to make the calibration using one tube for the blank containing broth, and reserving a second for the sample of culture. In every case the reading of the latter was preceded and followed by a reading of the blank, all three readings being repeated if necessary until the first and last were in agreement. Calibration was made with dilutions in broth of a formalinized culture of *E. coli* which had been incubated 24 hours and standardized by plate count. The turbidity of formalinized cultures did not change during several hours. The instrument was suitable for

measuring populations of 10^7 to 10^9 organisms of minimum size (10^6 to 10^8 in young cultures) per milliliter, giving values reproducible within less than two per cent in the region of 10^8 per milliliter. In the present experiments, cultures were diluted, if necessary, with broth to approximate this number before measurement.

Since the optical density of bacterial suspensions may vary with factors other than the size and number of organisms, preliminary experiments were necessary to ascertain the validity of interpreting as bacterial mass the turbidity of cultures of different age in terms of a single calibration. For this purpose, suspensions of organisms washed in saline were prepared from three-

TABLE 2

Comparison of optical density with nitrogen content of suspensions of young and old cells

AGE OF SOURCE CULTURE	VIABLE* COUNT (VC)	NEPHELOMET-RIC* COUNT (NC)	NITROGEN* PER ML. (N)	APPARENT SIZE (NC/VC)	RATIO (N/NC)
hours	$\times 10^6$ *per ml.*	$\times 10^6$ *per ml.*	*mgm.*		
24	940	835	0.025	0.9	0.30
3.5	310	739	0.025	2.4	0.34

* Cells washed twice and resuspended in sufficient saline to give a turbidity equal to 10^9 organisms per ml.

and-one-half and twenty-four hour cultures respectively, and the bacterial content of each was determined by parallel measurements of bacterial nitrogen and optical density. In addition the suspensions were counted to provide a measure of relative size.[2] Table 2 sets forth the results of this experiment, in which it can be seen that the ratio of turbidity to nitrogen is constant within expected error in spite of the three-fold difference in aver-

[2] Since measurements of size are based on viable counts, the values are valid only for suspensions of viable organisms. There is no indication that our 24-hour cultures contain appreciable numbers of non-viable cells (see, for example, an earlier experiment, Hershey, 1937). Nor is it probable that considerable numbers of cells from young susceptible cultures die during the process of washing, since in actual test cultures of this age diluted largely in saline were found to be 90 per cent viable after one hour at room temperature, in contrast to their mortality in water (table 1).

TABLE 3

Initial growth rates in broth at pH 7.4 of E. coli from cultures of different age

A. Nitrogen determinations

DATE OF EXPERIMENT	PARENT CULTURE			SUBCULTURE							
	Age	Viable count	Period of incubation	Initial* viable count	Final viable count	Initial* N/ml.	Final N/ml.	Initial N/cell	Final N/cell	Rate of multiplication	Rate of growth†
	hours	per ml.	hours	$\times 10^6$/ml.	$\times 10^6$/ml.	$\times 10^{-4}$ mgm.	$\times 10^{-4}$ mgm.	$\times 10^{-10}$ mgm.	$\times 10^{-10}$ mgm.	gen./hr.	per hr.
3/16/38	24	2.2×10^9	1.58	2.2	58.0	0.57	21.0	0.26	0.36	0.7	3.3
3/17/38	3	7.4×10^8	1.50	0.37	51.0	0.49	14.0	1.32	0.28	3.4	3.2

B. Manometric measurements

DATE OF EXPERIMENT	PARENT CULTURE			SUBCULTURE							
	Age	Viable count	Period of incubation	Initial* viable count	Final viable count	Initial* RO₂/ml.	Final RO₂/ml.	Initial RO₂/cell /hour	Final RO₂/cell /hour	Rate of multiplication	Rate of growth†
	hours	per ml.	hours	$\times 10^9$/ml.	$\times 10^8$/ml.	mm³/hr.	mm³/hr.	$\times 10^{-7}$ mm³	$\times 10^{-7}$ mm³	gen./hr.	per hr.
3/20/38	24	2.0×10^9	1.25	20.0	39.0	4.05	35.4	2.0	9.1	0.8	2.5
4/ 5/38	36	1.5×10^9	1.50	1.5	5.0	0.45	9.0	3.0	16.3	1.2	2.9
3/18/38	72	1.2×10^9	2.33	1.2	20.0	0.21	13.5	1.8	6.8	1.7	2.6
3/18/38	3.3	2.0×10^7	1.17	2.0	28.0	1.35	15.6	6.8	5.6	3.3	3.0

C. Nephelometric measurements

DATE OF EXPERIMENT	PARENT CULTURE			SUBCULTURE								
	Age	Viable count	Period of incubation	Initial* viable count	Final viable count	Initial§ nephelometric count	Final§ nephelometric count	Initial size¶	Final size¶	Rate of multiplication	Rate of growth||
	hours	per ml.	hours	$\times 10^6$/ml.	$\times 10^6$/ml.	$\times 10^4$/ml.	$\times 10^4$/ml.			gen./hr.	per hr.
2/24/38	24		3.00			0.1	79.0				3.1
3/13/38	24	1.3×10^9	2.50	0.13	4.5	0.2	41.0	1.4	9.1	2.1	3.1
3/16/38	24	2.2×10^9	1.58	2.2	4.0	2.1	53.0	1.0	6.0	0.7	3.1
4/ 5/38	36	1.5×10^9	1.50	1.5	5.5	1.7	32.0	1.2	6.0	1.2	2.8
3/18/38	72	1.2×10^9	2.33	1.2	20.0	2.0	97.0	1.6	4.8	1.7	2.4
4/ 5/38	24	1.9×10^9	0.75	9.7	9.3	7.5	34.0	0.8	3.7	0.0	2.9
3/20/38	24	2.0×10^9	1.25	20.0	39.0	16.0	18.0	0.8	4.5	0.8	2.8
3/21/38	24	5.0×10^9	0.50	50.0	41.0	47.0	80.0	0.9	2.0	0.0	2.1
3/24/38	36	1.0×10^9	0.50	26.0	26.0	28.0	64.0	1.1	2.5	0.0	2.4
3/ 3/38	3.0	1.3×10^7	2.00	0.13	17.0	0.8	80.0	6.1	4.7	3.5	3.3
3/17/38	3.0	7.4×10^6	1.50	0.37	6.8	2.9	51.0	8.0	4.5	3.4	2.8
3/18/38	3.3	2.0×10^7	1.17	2.0	28.0	9.7	110.0	4.8	3.9	3.3	3.0

* Values obtained by analysis of the parent culture, corrected for the dilution resulting when the subculture was seeded.

† Two-fold increases in bacterial nitrogen per hour.

‡ Two-fold increases in rate of O_2-use per hour.

§ Turbidity expressed in terms of calibration with 24-hour culture.

¶ Ratio of nephelometric to viable count (24-hour cells = ±1.0).

\|\| Two-fold increases in turbidity per hour.

age size of cells in the two suspensions. The nephelometric method is, therefore, suitable for the estimation of bacterial mass in cultures of different age, a conclusion which might also be made from the agreement between growth rates obtained by the various methods (table 3).

The method used for determining bacterial nitrogen has already been described (Hershey, 1937). The principal source of error with this method is in centrifugation. Recentrifuging in conical tubes is essential. Populations of 10^7 per ml. or greater may be satisfactorily determined, the micro-Kjeldahl method requiring about 200 ml. of the three-hour cultures, and as little as 10 ml. (0.3 mgm. N) of cultures fully grown.

The use of the Warburg-Barcroft manometer in our hands has been referred to (Hershey and Bronfenbrenner, 1937 and 1938). Bacteria in young cultures were estimated by measuring the oxygen consumption of five ml. aliquots without added nutrient, after determining that the oxygen use under these conditions was not different from that in fresh broth. This has been found to hold substantially true for cultures in which the population has not reached 10^9 organisms per milliliter. For the estimation of bacteria in older cultures, one ml. of the uncentrifuged culture was placed in the side arm of the manometer vessel, with 4 ml. of broth on the floor. In either case, after temperature equilibration, the side-arm was tilted and the oxygen consumption at zero time obtained in the usual way. This method was applicable to measurement of 10^8 or more organisms of minimum size per milliliter. Values obtained in this way represent, of course, the bacteria present at the time of reading, rather than at the time of removal from the parent culture.

NEPHELOMETRIC MEASUREMENT OF RATE OF GROWTH

Broth in 100 ml. amounts, filtered asceptically through paper into a one-liter Erlenmeyer flask, was warmed to 37°C. in a water bath and seeded with the desired aliquot of the parent culture. The latter was prepared as previously described from a seeding of 10^5 organisms per milliliter. If the final population for the

growth rate measurement was to exceed 10^8 organisms per milliliter, aeration was practised to avoid suppression of growth due to lack of oxygen. Immediately after making the transfer, the parent culture was counted, and directly killed by addition of 0.5 per cent formalin. Turbidity of the formalinized sample was measured within 10 minutes. At the end of the incubation period the subculture was analyzed in the same way. From the values obtained for the two cultures, the rate of growth was expressed by the formula $n/t = \dfrac{\log b - \log B}{t \log 2}$, where n/t is the reciprocal of the apparent generation time, B is the initial and b the final number of bacteria, and t the period of incubation, these values referring to the daughter culture. Substitution into this formula of the viable counts gave the rate of multiplication in generations per hour, while the nephelometric values gave the rate of mass increment.[3] Since the turbidity of young cultures is slightly below the value expected from the nitrogen content (table 2), the tendency of error from this source is to give abnormally low values for the growth rates of old cells. The ratio of the nephelometric to the viable count was taken as the index of size. The nephelometer having been calibrated with 24-hour cultures, the index is near unity for cultures of this age and proportionately larger for the young cultures, with a maximum value approaching ten.

MEASUREMENT OF RATE OF GROWTH BY DETERMINATION OF BACTERIAL NITROGEN

This method has been used primarily as a control corroborating nephelometric experiments. The procedure described above was followed exactly, with the exception that nephelometric measure-

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[3] The growth rate calculated from nephelometric data may be defined as the number of two-fold increases in turbidity per hour, and would be identical with the multiplication rate (generations per hour) in a hypothetical culture in which the average size of cells remained constant. This unit has been chosen to give values numerically comparable with the multiplication rate. Values given by the formula we have used can be readily converted to those commonly expressed by the equation $C_v = 1/t \ln b/B$ to which they are proportional, by applying the factor 0.69.

ments were supplemented by determinations of the nitrogen in the washed sediment from the formalinized cultures. Growth rates were computed in the same way as from the nephelometric data. Size was expressed as nitrogen per viable cell, which was found to be about 0.3×10^{-10} mgm. for 24 hour cultures, with a maximum of 10 times this figure for young cultures.

MANOMETRIC MEASUREMENT OF GROWTH RATE

This method provided a valuable check on the two preceding methods, since it is not subject to possible errors due to formation of non-bacterial sediments in the medium. A systematic error is introduced, however, from the fact that removal of carbon dioxide in the respirometer vessel depresses the rate of growth during the interval of equilibration of temperature. This effect tends to cancel out when young cultures are being studied, and to give somewhat low values for the growth rate of cultures seeded with old cells. The depressive effect of lack of carbon dioxide on the growth rate was found to be less marked the larger the numbers of bacteria in the vessel, and introduced no significant error under the conditions used when 10^8 or more bacteria per ml. were present.

The manometric measurement of growth rate differed in detail from the two preceding methods. Aliquots of the parent culture representing 10^8 to 10^9 bacteria each were placed in duplicate vessels of the respirometer and the rate of oxygen consumption determined after equilibration of temperature. The bacterial count was made and the seed introduced into the subculture at the moment when reading of the manometers was begun, samples being taken for this purpose from the duplicate vessel. After a suitable incubation period, as the numbers approached 10^8 bacteria per ml., five ml. aliquots of the daughter culture were placed in each of the two warmed vessels and equilibrated 20 minutes in the water bath. When the temperature was constant, the viable count was made from the duplicate vessel, and readings were begun as before. The period of incubation of the subculture was thus represented by the interval between the zero times of the respective measurements. The

growth rate was obtained by substitution of these values into the formula. Size, expressed as rate of oxygen uptake per cell, was found to be about 2.0×10^{-7} mm³ per hour for 24-hour cultures, and five to eight times greater in young cultures. Maximal size is difficult to observe by this method, for the reason that the cells are already becoming smaller when the bacteria are numerous enough to permit of measurement, but the values conform well with indices obtained at corresponding times by the other methods.

Values for the rates of multiplication and of growth determined by the three methods, together with data on population and average size in the cultures studied, are given in tables 3 and 4. Reference to the last column of table 3A, B and C, showing the rate of growth obtained by the various methods, discloses at once the substantial agreement.

In table 5 the observed values have been averaged and submitted to statistical analysis. Transplants made from the young cultures have shown only insignificantly higher rates of growth, the odds against these differences resulting from chance being about three to one and two to one, respectively, for the two experimental series. The ratio of the difference to its standard error for the first series is 1.1, for the second 0.8, while the minimum significant value is generally taken to be 3.0. On statistical reasoning alone, therefore, neglecting the tendency of known experimental errors, the smallest difference in growth rate to be regarded as significant is 0.36 generation per hour for the second series. Thus, with the average value of 21.4 minutes for the apparent generation time of old cells, that of more rapidly growing young cells should be at most 19.0 minutes. Actually, the latter value is 20.7 minutes. It will be remembered, moreover, from the discussion of the several methods, that the known errors tend in each case to give abnormally low values to the growth rate of the elder seedings, and the presence of dead cells in these seedings undoubtedly augments this tendency. The difference actually observed, about six per cent for the totaled values, is entirely within the limits of magnitude reasonably attributable to these sources of error.

TABLE 4

Initial growth rates in broth at pH 6.8 of E. coli from cultures of different ages

Nephelometric measurements

| | PARENT CULTURE | | | SUBCULTURE | | | | | | | | |
DATE OF EXPERIMENT	Age	Viable count	Period of incubation	Initial* viable count	Final viable count	Initial§ nephelometric count	Final§ nephelometric count	Initial size ¶	Final size ¶	Rate of multiplication ¶	Rate of growth‖
	hours	per ml.	hours	×10⁹/ml.	×10⁹/ml.	×10⁹/ml.	×10⁹/ml.			gen./hr.	per hr.
4/ 8/38	24	1.9×10^9	2.50	0.2	5.5	0.21	35.0	1.1	6.3	1.9	3.0
4/14/38	24	9.1×10^8	2.91	0.1	8.5	0.15	44.0	1.4	5.2	2.2	2.9
4/ 9/38	24	1.9×10^9	1.40	1.9	3.0	2.1	22.0	1.1	7.3	0.5	2.5
4/15/38	24	1.2×10^9	1.40	1.2	1.4	1.4	17.0	1.2	12.1	0.2	2.6
4/16/38	24	1.1×10^9	1.66	1.1	3.1	0.9	22.0	0.8	7.1	0.9	2.8
4/16/38	48	1.6×10^9	1.78	1.6	5.4	1.8	32.0	1.1	6.0	1.0	2.4
4/11/38	12	8.5×10^8	0.66	8.5	7.3	8.8	35.0	1.0	4.8	0.3	3.0
4/13/38	12	7.9×10^8	0.66	7.9	9.6	8.8	30.0	1.1	2.5	0.4	2.6
4/12/38	24	1.3×10^9	0.33	6.5	6.5	5.7	12.0	0.9	1.8	0.0	3.2
4/12/38	24	1.3×10^9	0.66	6.5	6.8	5.7	25.0	0.9	3.7	0.1	3.2
4/13/38	24	1.1×10^9	0.33	5.4	4.7	6.6	12.0	1.2	2.6	0.0	2.6
4/14/38	24	9.6×10^8	0.43	9.6	9.0	12.0	28.0	1.2	3.1	0.0	2.8
4/12/38	3.0	5.5×10^5	3.40	0.01	1.2	0.07	14.0	6.0	11.7	2.0	2.6
4/14/38	3.2	9.3×10^5	2.75	0.03	8.8	0.15	35.0	5.3	3.9	3.0	2.9
4/13/38	3.5	3.2×10^7	2.53	0.06	7.4	0.26	42.0	4.1	5.7	2.7	2.9
4/11/38	2.5	2.2×10^6	1.33	0.22	4.2	1.55	27.0	7.0	6.5	3.2	3.1
4/17/38	2.5	3.1×10^6	1.33	0.31	5.3	2.00	29.0	6.5	5.5	3.1	2.9
4/17/38	3.0	6.6×10^6	1.33	0.33	5.2	2.00	30.0	6.1	5.8	3.0	2.9
4/15/38	3.5	2.3×10^7	1.66	0.23	3.3	1.01	20.0	4.4	6.1	2.3	2.6
4/ 9/38	3.5	3.0×10^7	0.45	3.0	7.3	12.4	36.0	4.1	5.0	2.9	3.4
4/15/38	3.5	2.3×10^7	0.66	2.3	7.0	10.1	30.0	4.4	4.3	2.4	2.4

* § ¶ ‖ See table 3-c.

If it be agreed that no significant differences exist between the growth rates of young and old bacteria, the same cannot be said of the rates of multiplication. The latter, calculated for the same cultures (tables 3 and 4), illustrate very clearly the marked differences corresponding with the age of the inoculum which are commonly observed. The paradox that old and young bacteria may exhibit identical growth rates, while differing markedly in rate of multiplication, is to be explained only by consideration of average cell-size. Growing at the same rate, the smaller bacteria from old cultures require a longer time

TABLE 5

*Effect of pH and age of culture on rate of growth of transplants of E. coli**

NUMBER OF OBSERVATIONS	pH	AGE OF PARENT CULTURE	GROWTH RATE	APPARENT GENERATION TIME	DIFFERENCE	STANDARD DEVIATION	STANDARD ERROR OF MEAN	STANDARD ERROR OF DIFFERENCE	RATIO OF DIFFERENCE TO S. E.	ODDS AGAINST DIFFERENCE DUE TO CHANCE
		hours	*per hour*	*minutes*	*per cent*					
13	7.4	12–72	2.8	21.4	} 10.0	0.338	0.094	} 0.270	1.1	3:1
5	7.4	2– 3.5	3.1	19.3		0.566	0.253			
12	6.8	12–72	2.8	21.4	} 3.3	0.257	0.074	} 0.118	0.8	2:1
9	6.8	2– 3.5	2.9	20.7		0.283	0.094			

* Analysis of data of tables 3 and 4.

("lag") to reach the size at which fission occurs in freshly seeded broth, than do young bacteria of greater initial size.

Inspection of the tables reveals no systematic errors affecting the conclusions to be drawn. The two series of experiments, using broth of pH 6.8 and 7.4 respectively (table 5), have yielded identical results. The somewhat smaller experimental variation in the latter series bespeaks the accuracy which comes with practice.

It should be observed that the experiments include a rather wide range of initial seedings, and periods of incubation, neither of which has had any effect on the rate of growth. This adds considerably to the significance of the findings, for the magnitude and kind of errors expected under the several conditions

are very different. The smaller seedings were used especially to test the argument that differences in growth rate of organisms of different age might be magnified in this case, as are differences in the rate of multiplication. On the other hand, with large seedings growth could be measured over a much shorter time period, even a fraction of a single generation time, which might be expected to consist quite neatly of the period of "rejuvenescence." No sign of lag in absolute growth rate was encountered, however, in any of the experiments, the recorded values agreeing precisely with the maximum rates of growth attained in cultures observed continuously by the same methods (unpublished data). The initial growth rates measured in these experiments represent, therefore, the maximum rate for this organism under the conditions of cultivation employed, and no "period of adjustment," either of bacteria to the new medium or the reverse, is evident. The fact that the maximum absolute rate of growth is less than three "doublings" per hour, while the rate of multiplication at its maximum may exceed four generations per hour, is explained by the circumstance that the latter is observed during the period in which size is decreasing, i.e., bacteria are actually multiplying faster than they are growing.

The bearing of these observations on the general problem of growth phases is clear: that so far as differences in rate of numerical increase are concerned, "physiological youth" of the cells in young cultures of *E. coli* is an artifact, and that changes in size, conditioned in turn by changing environmental status, and the direct effect of the latter on metabolism, sufficiently explain the course of events in the development of the culture. Experiments are being planned, directed toward further elucidation of these relationships.

SUMMARY

Initial growth rates deduced from measurements of turbidity, bacterial nitrogen, and oxygen uptake of broth transplants of *Escherichia coli* seeded from young and old cultures respectively, have given identical values, while parallel measurements of rate of multiplication revealed the expected differences. We con-

clude that the physiologic state of the cells does not influence their rate of growth, and that the lag in rate of multiplication must be attributed to changes in the factors limiting the size at which fission occurs during the period of growth.

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A STUDY OF THE LIPIDS OF CERTAIN ENTERIC BACILLI[1]

CLARA H. WILLIAMS, WALTER R. BLOOR, AND LESLIE A. SANDHOLZER

Departments of Biochemistry and Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, New York

During the past decade bacteriologists have become increasingly interested in the study of the chemical composition of bacterial cells. Numerous investigators have demonstrated a correlation between the cell components and certain biological activities. Most of the investigations have been concerned with carbohydrates, proteins, nucleic acids, *etc.*; the lipid fractions have been only slightly studied. Thus, there is a need for an intensive study of the bacterial lipids. The present paper is the first of a series of such studies.

LITERATURE

In 1893 Cramer demonstrated that the lipid content of certain common organisms varied with the carbohydrate content of the nutrient medium. Using a Friedländer's bacillus, he found that the lipid content of the organism was 10.26 per cent on nutrient agar, but on the same medium plus 5 per cent glucose, the lipid content rose to 22.68 per cent. Lyons (1897) verified this work by showing that the lipid content of an organism isolated from water, when grown on a one-per-cent glucose medium, had a lipid content of 11 per cent, but when the amount of glucose was increased to 10 per cent, the lipid content rose to 23 per cent. Nishimura (1893) also studied an organism isolated from water

and found the total lipid to be 3.19 per cent of the dry weight of the bacteria. Approximately 0.68 per cent of the dry weight of the organism was lecithin. He isolated a fatty acid which melted at 15°C. and a mixture of fatty acids which melted at 51°C. The former, he believed to be oleic acid, the latter a mixture of palmitic and stearic acids.

Nicolle and Allilaire (1909) analyzed a series of enteric bacilli grown upon a potato agar at 37°C. for 24 hours. The total lipid content ranged from 9 to 16 per cent of the dry weight of the organisms and the presence of phosphorus in the extracts indicated that a phospholipid was probably present. Dawson (1919) found that the lipid content of *Escherichia coli* did not vary appreciably when he employed various combinations of peptone, meat extract, edestin, flour proteins, and potato juice in the nutrient media, except when one per cent glycerol was incorporated. The total lipid on the glycerol-free media varied from 4 to 5 per cent, but in the presence of glycerol it rose to 8 per cent.

Eckstein and Soule (1931) grew *Escherichia coli* on a synthetic medium containing alanine in one case and cystine in another as the source of nitrogen. In the former the lipid content of the dry cells was 7.8 per cent, in the latter the lipid content dropped to 3.6 per cent. The iodine number of the total lipid was 25.6 and that of the total fatty acids 37.4. They postulated, therefore, that oleic acid was the only unsaturated fatty acid present. They reported that a phospholipid was present to the extent of 17.4 per cent of the total lipid, but no cholesterol or other sterols were found. The absence of sterols had been observed by Miyoshi (1930). Hecht (1935), on the other hand, reported that he obtained a positive test for cholesterol with the residue, when he warmed petroleum-ether extracts of *Escherichia coli* for 8 hours.

EXPERIMENTAL

Materials and methods

Organisms. Nine strains of Gram-negative, non-sporulating, enteric bacilli were used for analysis. They represented four species or types. Of these, four strains, 214, 214PA5, 225, and

229B, were *Escherichia coli*; three strains, 234, 239-1 and 248-1, were *Escherichia communior*; one strain, 300-1, was a characteristic *Escherichia-Aerobacter* "intermediate", strain 691, (Tittsler and Sandholzer, 1935); and strain 657 was *Shigella paradysenteriae* (Flexner).   Originally, strain 229B fermented sucrose, but during the past year it has lost this capacity.   Organism 214PA5 is a substrain of 214.   It was isolated after several serial passages of the parent strain on lithium-chloride agar (nutrient agar plus 0.3 per cent lithium chloride) in July 1936.   Since then it has been maintained on plain agar.

Culture media.   Throughout the present study, the organisms used for analysis were grown on plain agar having the following composition: peptone (Bacto), 20 grams; beef extract (Bacto), 3 grams; sodium chloride, 5 grams; water (distilled), 1000 cc.; final pH, 7.4 to 7.6.   The agar was dispensed in Kolle flasks and Petri dishes.

The peptone was analyzed carefully for the presence of lipid. Calculations based on the analysis of 15 grams of dry peptone showed that it contained less than 0.01 per cent of total lipid. Analyses reported in the literature, (Leach and Winton, 1920), for beef extract show that it contains approximately 0.16 per cent total lipid, an amount which is negligible for the present purpose.

Stock cultures for inoculation of the agar were prepared in a medium of the following composition: peptone (Bacto), 20 grams; sodium chloride, 5 grams; water (distilled), 1000 cc.; final pH 7.4 to 7.6.

An agar medium was chosen in preference to a liquid one for growing the organisms for analysis because of the greater ease with which the organisms could be harvested in large quantities. The usual harvest of dry bacteria was approximately 2 grams from a total of 79 containers (44 Kolle flasks and 35 Petri dishes), or 2.8×10^{-3} mgm. per' sq. mm.

Preparation of samples for lipid analysis.   The surface of the agar medium was inoculated with a 24-hour peptone-water culture of the organism and incubated at 37°C. for 24 hours. The consequent growth was washed from the surface of the agar with sterile distilled water and the organisms were sedi-

mented by high speed centrifugation for an hour at approximately 2500 r.p.m. The sedimented residue was then frozen and dried in the frozen state *in vacuo*. The viable organisms dried in this manner were used for analysis.

Extraction of the lipids. The dry organisms were ground by hand in a mortar to a fine powder and aliquots of from 1 to 2 grams were carefully weighed in a 22 × 80 mm. extraction thimble. Extraction of the lipids was carried out using, first, alcohol (2 parts) and later ethyl ether (1 part). To shorten the time required for extraction, and to make the extraction more complete, hot alcohol was employed. The thimble was inserted in a tower above a flask containing the boiling solvent and the lipids were thus extracted continuously with fresh hot alcohol.

Determination of lipid fractions. Suitable aliquots of the alcohol-ether extract were used for determining the total fatty acids, the phospholipid, the fatty acids in the phospholipid by the oxidative methods of Bloor (1928). The iodine numbers of each of these fractions were determined according to the method of Yasuda (1931).

The percentage of phosphorus in the phospholipid was found by digesting the phospholipid with 10 N sulfuric acid and Superoxol and measuring the amount of phosphate in the resulting solution using the colorimetric method outlined by Kuttner and Cohen (1927). The percentage of nitrogen was determined by digesting the phospholipid as in the case of phosphorus and determining the ammonia formed by Nesslerization as suggested by Folin and Farmer (1912). The method was perfected, so that as little as 20 to 40 gamma could be determined by the use of a sensitive photoelectric colorimeter developed by Bloor (1938).

Qualitative tests for cholesterol and other sterols were made on the acetone soluble portion of the total lipid using the Liebermann-Burchard and the Windaus method modified by Okey (1930).

Results

The detailed data of the lipid analyses expressed as per cent dry weight of the bacteria are presented in tables 1 to 5 inclusive,

and will be discussed below under separate headings.   Most of the findings are summarized graphically in figure 1.   Sufficient data have been presented in each table to permit the reader to evaluate for himself the significance of the differences between organisms.

Total fatty acids.   The values for total fatty acids and the respective iodine numbers are given in table 1.   The data show that the total fatty acids varied from approximately 2.5 to 5.0 per cent of the dry weight of the bacteria.   No correlation was
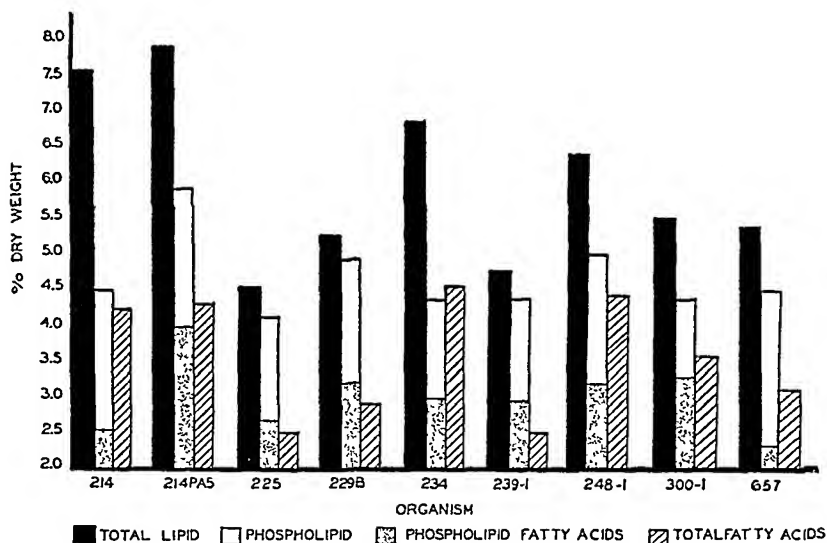
FIG. 1. SUMMARY OF ANALYSES OF BACTERIAL LIPIDS

noted between the total fatty acids and the cultural characteristics of the organisms.   In general, the organisms fall into two groups, however, those having total fatty acids comprising from 4 to 5 per cent of the cell (214, 214PA5, 234, 248-1) and those whose total fatty acids represent from 2.5 to 3.0 per cent of the cell, (225, 229B, 239-1, 657).   The single *Escherichia-Aerobacter* "intermediate" strain in the group has a total fatty acid content which is midway between the two groups.

The iodine numbers show a wide, uncorrelated variation, with values ranging from 42 to 82.   The values are all below that of oleic acid which has an iodine number of 90.   It is probable

that the only unsaturated fatty acid present is oleic, and it is probably present in varying amounts.

TABLE 1

Analysis of bacterial lipids. Total fatty acids based on dry weight and the corresponding iodine numbers

ORGANISM	PER CENT TOTAL FATTY ACIDS				IODINE NUMBERS			
	Number of determinations	Mean	Standard deviation	Range	Number of determinations	Mean	Standard deviation	Range
214	6	4.21	0.10	4.01–4.45	3	73	1.7	71–75
214PA5	4	4.27	0.10	4.14–4.38	4	48	3.7	42–52
225	3	2.51	0.14	2.47–2.56	4	49	1.0	48–50
229B	4	2.86	0.17	2.71–3.02	3	53	1.4	50–54
234	3	4.60	0.47	4.26–4.89	3	62	3.7	52–70
239-1	4	2.62	0.26	2.47–2.70	3	82	9.4	71–94
248-1	3	4.41	0.10	4.30–4.51	3	42	3.6	39–47
300-1	3	3.43	0.22	3.40–3.46	3	76	0.0	73–78
657	3	2.92	0.26	2.87–2.99	3	59	6.8	57–63

TABLE 2

Analysis of bacterial phospholipids. Phospholipid based on dry weight and the corresponding iodine numbers

ORGANISM	PER CENT PHOSPHOLIPID				IODINE NUMBERS			
	Number of determinations	Mean	Standard deviation	Range	Number of determinations	Mean	Standard deviation	Range
214	4	4.36	0.33	3.90–4.65	3	70	10.3	61–80
214PA5	4	5.66	0.33	5.51–5.85	3	52	7.2	47–57
225	3	4.07	0.10	4.02–4.14	4	47	12.0	47–50
229B	3	5.04	0.14	4.83–5.15	3	31	0.0	29–32
234	4	4.48	0.26	4.24–4.69	3	41	5.3	41–42
239-1	3	4.35	0.00	4.15–4.56	4	27	3.6	23–32
248-1	4	4.89	0.28	4.78–5.01	3	87	3.7	85–88
300-1	3	4.87	0.24	4.81–4.92	3	60	6.5	59–62
657	3	4.40	0.22	4.29–4.58	3	75	7.2	73–76

Phospholipids. The values for the phospholipid content of the dried bacteria and the corresponding iodine numbers are recorded in table 2. Apparently the phospholipid represents the major portion of the bacterial lipid. It ranges from approxi-

mately 4 to 6 per cent in this group of organisms, the total lipid varying from 4.5 to 8 per cent (table 6). The phospholipids appear to be fairly constant for the organisms.

The variation in iodine number, which ranges from 23 to 88, may have two explanations. There may be a wide variety of phospholipids present which would account for such variations, or there may be but one phospholipid, having the same degree of unsaturation, but present in varying amounts.

Phospholipid fatty acids. The data presented in table 3 indicate that the phospholipid fatty acids in these organisms are

TABLE 3

Analysis of bacterial phospholipids. Phospholipid fatty acids based on dry weight and the corresponding iodine numbers

ORGANISM	PER CENT FATTY ACID IN PHOSPHO-LIPID	PER CENT PHOSPHOLIPID FATTY ACID				IODINE NUMBERS			
		Number of determinations	Mean	Standard deviation	Range	Number of determinations	Mean	Standard deviation	Range
214	63	3	2.64	0.57	2.65–2.75	4	32	7.6	30–35
214PA5	68	3	3.94	0.26	3.80–4.07	2	36	2.0	34–38
225	67	3	2.72	0.14	2.68–2.78	3	67	3.5	64–68
229B	64	3	3.21	0.17	3.18–3.28	3	33	5.4	31–37
234	64	4	2.90	0.24	2.69–3.08	3	60	6.3	52–65
239-1	73	3	3.18	0.10	2.97–3.37	4	47	3.5	43–50
248-1	67	4	3.27	0.01	3.13–3.42	3	77	3.2	76–77
300-1	65	3	3.26	0.14	3.22–3.34	3	50	6.5	47–54
657	61	3	2.36	0.01	2.31–2.44	3	86	3.6	83–91

fairly constant in amount and normal when compared to the values for all the known phospholipids.

The amount of phospholipid fatty acids in the dried bacteria is approximately 3 per cent. The iodine numbers also indicate here that the amount of unsaturated acid present is small. If one compares the iodine number of the phospholipid with that of the phospholipid fatty acid, it will be noted that with bacterial strains 214, 214PA5, 248-1, 300-1, the iodine number of the phospholipid is higher than that of the phospholipid fatty acid. Theoretically this is not possible. It has been observed by other workers, however, and has been explained by the hypothesis

that some oxidation of the unsaturated fatty acids takes place on separating the fatty acids from the phospholipid molecule.

Total lipid. The total lipid is a calculated value arrived at by adding the values for the neutral fat, the phospholipids, and the non-saponifiable portion of the alcohol-ether extract. By subtracting the value for the phospholipid fatty acids from the value for total fatty acid, the percentage of fatty acid present in the organisms as neutral fat is obtained. The non-saponifiable portion usually contains the sterol fraction. These values are shown in table 4.

TABLE 4

Analysis of bacterial lipids. Total lipid, non-saponifiable matter and neutral fat fatty acids based on dry weight

ORGANISM	CALCULATED AVERAGE TOTAL LIPID	CALCULATED AVERAGE NEUTRAL FAT FATTY ACIDS	NON-SAPONIFIABLE MATTER*
214	7.36	1.57	1.43
214PA5	7.91	0.43	1.82
225	4.45	0.00	0.38
229B	5.27	0.00	0.23
234	6.88	1.68	0.72
239-1	4.66	0.00	0.31
248-1	6.44	1.14	0.41
300-1	5.44	0.17	0.40
657	5.36	0.56	0.40

* Based on one determination only.

The total lipid in these organisms varies from approximately 4.5 to 8.0 per cent, these variations showing no correlation with the species or type. The amount of neutral-fat fatty acids also shows uncorrelated variations and ranges from zero to almost 2 per cent. The apparent lack of neutral-fat fatty acids in some cases indicates that the only fatty acids present are in the phospholipid.

The non-saponifiable portion of the lipid represents, for the most part, a very small fraction. Only strain 214 and its substrain 214PA5 show appreciable amounts. Just what this non-saponifiable portion represents is not known, since the amount available was not sufficient to permit further tests to be made

upon it. Ordinarily this portion would represent the sterol fraction of the total lipid from blood, tissues, *etc.*, but the organisms investigated contained no measurable amounts of any sterol.

Phosphorus and nitrogen in the phospholipid. To determine whether the phospholipids of the enteric bacilli are similar to those commonly found in animal and plant tissues, phosphorus and nitrogen determinations were made. Such analyses indicate the type of phospholipid concerned. The amount of phosphorus in ordinary lecithin and cephalin ranges from 3.75 to 4.25 per cent, depending upon the kind of fatty acid present in the molecule, while the nitrogen content ranges from 1.72 to 1.92 per

TABLE 5

Analysis of bacterial phospholipids. Phospholipid phosphorus and nitrogen

ORGANISM	AVERAGE PER CENT OF PHOSPHOLIPID		ATOMIC PHOSPHORUS-NITROGEN RATIO
	Phosphorous	Nitrogen	
214	4.40	0.00	1:0
214PA5	2.77	1.84	1:1.3
225	3.41	2.89	1:1.8
229B	3.41	1.66	1:1.1
234	3.11	2.03	1:1.5
239-1	4.66	1.28	1:0.5
248-1	3.51	1.74	1:1.1
300-1	3.45	2.33	1:1.5
657	3.68	1.40	1:0.9

cent. Chibnall and Channon (1927) described another type of phospholipid present in the leaves of the cabbage plant, and named it phosphatidic acid. It differs from the ordinary phospholipid in that it contains no nitrogen base, calcium or magnesium replacing this substance in the molecule. The percentage of phosphorus and nitrogen and the ratio of one to the other, therefore, aid in interpreting the type of phospholipid present.

The results of the analyses for phosphorus and nitrogen in the phospholipids of the bacteria are presented in table 5. The amount of phosphorus in the phospholipid is low, except for strains 214 and 239-1, in which it is higher than normal. The nitrogen values vary within the group some being higher than

usual and others lower. The results obtained with strain 214 are interesting since it is the only one that gave no yield of nitrogen in the phospholipid. When nitrogen is lacking, the percentage of phosphorus in the molecule is higher. The value for phosphorus in strain 214 agrees with this. A phosphatidic acid containing calcium in the molecule would theoretically have 4.48 per cent of phosphorus and no nitrogen. The phosphorus and nitrogen values of strain 214 coincide with those of such a phosphatidic acid. The phosphorus value for strain 239-1 is high, but the nitrogen content is normal, hence a phosphatidic acid is probably absent or is present only in small amounts.

The atomic ratio of phosphorus to nitrogen, with the exception of cultures 214 and 239-1, is approximately 1:1. Most of the values are slightly higher than this, probably because of some extraneous nitrogen. It has been found by MacLachlan (1935) and others that unless the phospholipid is carefully purified by dissolving it in water and precipitating it with acetone, the per cent of nitrogen will always be higher than the calculated theoretical values. Since there is a considerable loss of phospholipid in this procedure, the amounts of phospholipid available for the present study did not warrant its use. Nevertheless, the evidence is fairly conclusive that the phospholipids found in these organisms resemble those of animal and plant tissues with respect to the content of nitrogen and phosphorus.

No evidence of cholesterol or any other sterol in the lipids of the bacteria was found, *i.e.*, it was calculated to be present in amounts less than 0.3 per cent of the dry weight of the bacteria.

DISCUSSION

Although the total lipid of certain enteric bacilli is approximately what one might expect with other organisms, these bacteria are peculiar in two respects. First, the greatest portion of the total lipid is phospholipid. The significance of this finding is not clear. Possibly the phosphorus metabolism of the organisms is linked with the lipid metabolism, but to date there is no evidence to support such a view. In fact, the reports in the

literature seem to indicate that the phosphorus in the phospholipid represents only a small fraction of the total phosphorus in the bacterial cell. The second peculiarity is the absence of sterol. This absence has been observed in the acid-fast bacilli by Anderson *et al.* (1935). These compounds are apparently not synthesized in the organism in detectable amounts or they are rapidly broken down and thus are not detectable because they are not stored in the organism.

The differences between strains 214 and 214PA5 offer an interesting problem since the only difference between these two organisms is that the latter was grown for several serial passages on lithium-chloride agar. The analyses of these two cultures agreed quite well with respect to the total fatty acid content, but there was no agreement in the iodine numbers of their fatty acids. Of all the strains studied, the 214PA5 strain had the highest phospholipid content. Again, the iodine numbers of the phospholipid from the substrain 214PA5 and the parent strain 214 did not agree. The same situation existed in the case of the phospholipid fatty acids and the corresponding iodine numbers. The neutral fat fatty acids were much higher in strain 214 than in 214PA5. The most striking difference which occurred, however, was in the nitrogen content of the phospholipid of these strains. Whereas 214PA5 had a normal complement of nitrogen in the phospholipid, 214, the parent strain, had a phospholipid which was nitrogen free. It hardly seems reasonable that the serial passage on lithium-chloride agar could have induced such marked differences in the metabolism of the organisms, particularly when one realizes that this strain had been serially transferred on plain agar for over a year.

SUMMARY

1. An analysis of the lipids of nine strains of enteric bacilli has been made. The total lipid content of the organisms varied from 4.3 to 7.9 per cent of the dry weight. No cholesterol or other sterols were found.

2. The total fatty acid content varied from 2.5 to 4.6 per cent of the dried bacteria. The iodine numbers were low, varying from 42 to 82.

3. Phospholipids constituted the major portion of the total lipids. With one exception they represented about 60 per cent of the total lipid.

4. The fatty acid in the phospholipid molecule ranged from 63 to 73 per cent. As indicated by their iodine numbers, the fatty acids were not all alike.

5. The content of phosphorus and nitrogen of the phospholipid was normal, with the exception of a single strain which showed evidence of a phosphatidic acid in place of a phospholipid.

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THE CULTURAL AND ANTIGENIC PROPERTIES OF SHIGELLA SONNEI

JOHN H. GLYNN AND D. HUGH STARKEY

Department of Bacteriology and Immunity, McGill University and the Royal Victoria Hospital, Montreal

The present study aims to define the taxonomic position of that group of dysentery bacteria commonly known as *Shigella paradysenteriae*, var. *sonnei* (Bergey, 1934). Most of the material on which this work is based has been recently isolated in this laboratory but a number of type strains of the species from The National Collection of Type Cultures in London, England, have been included for comparison. Also included for comparative purposes, are a number of strains of closely related members of the Genus *Shigella* which are mannitol-fermenters; namely, the groups known as Flexner, Dispar and Alkalescens.

ORIGIN OF STRAINS

A total of eighty-eight strains were studied. These included:

42 strains of *Shigella sonnei* isolated in this laboratory either within three months of the initiation of this work or while the work was in progress. All of these strains were recovered from the stools of patients with intestinal complaints;

12 strains of *Shigella sonnei* obtained from the National Type Collection at the Lister Institute, London;

2 strains of *Shigella alkalescens* obtained from Professor E. G. D. Murray both of which were original strains described by Sir Frederick Andrewes (1918);

3 strains of *Shigella dispar* isolated in this laboratory;

8 type strains of *Shigella dispar* obtained from the National Collection of Type Cultures at the Lister Institute, London.

4 strains of *Shigella dispar* obtained from Professor Murray

and originally described by him (1918) as his Class 4. At that time he identified them as the type described by Kruse (1907) as Type E, although he states (p. 387) "Class 4 is certainly equivalent to Kruse E.... The strains comprising Class 4 (Kruse E) would fall into the group comprised by Andrewes' *B. dispar*." Kruse E is at present generally held to be the same as *Shigella sonnei* (Gardner (1929) Gay (1935)).

5 strains of *Shigella paradysenteriae (flexner)* obtained from Professor Murray, representing the types V, W, X, Y and Z of Andrewes and Inman (1919) each of which is described in their monograph.

A. CULTURAL PROPERTIES

1. Colony form

The colony form was studied on plain agar, on Chandelier and on MacConkey plates. In general the Sonne strains produce colonies which are slightly larger, more granular and more opaque than those of the Flexner, Alkalescens and Dispar strains. All plates were kept in a moist jar for three weeks at 37°C.

a. Shigella sonnei colony. On original isolation all strains showed a uniform colony. At twenty-four hours incubation they were rather flat, slightly granular and opaque, about 3 to 4 mm. in diameter, with a smooth raised central zone grading out to a thin slightly irregular edge. The whole colony was lactose-negative. The colonies continued to increase in size and to spread peripherally until discarded and have reached three centimeters in diameter.

On further incubation, secondary colonies or papillae appeared on the surface of most, but not all, of the original colonies. These papillae, or daughter colonies, consisted of raised, smooth, entire, rounded outgrowths on the surface which, soon after their appearance, caused the medium immediately beneath them to show the lactose-positive change. These daughter colonies were usually 1 to 2 mm. in diameter within forty-eight hours after their appearance; some continued to increase in size while others did not. The length of time required for these papillae to appear varied from strain to strain but could be correlated roughly with

the time required for the individual strain to ferment lactose in peptone water medium.

Only one strain (out of fifty-four) failed to show secondary colonies.   In this strain the centre of the colony became progressively more heaped up and irregular and eventually the entire colony went lactose-positive.   Repeated platings of this strain failed to change its behavior.

Attempts were made to get these secondary papillae to breed true by carefully fishing them from the parent colony and replating through 10 to 12 generations.   Each generation began as a lactose-negative colony upon which lactose-positive papillae appeared at about the same interval as on original isolation. The twelfth generation did not differ from the first generation even on a percentage basis.   Similarly, the few original primary colonies which showed no lactose-positive papillae (although most other colonies of these same strains on the original plate did show numerous papillae) were replated up to twenty generations, alternating solid and fluid media, without changing the general appearance of any generation.   The colonies always started as lactose-negative and most of them later developed lactose-positive papillae.

A second colony form appeared on plating out from older cultures or from cultures grown in 1:10,000 Brilliant-Green Peptone Water.   This is a flat, entire colony with a bevelled edge, not granular and with a moist shiny surface, smaller than the ordinary colony, increasing little in size with further incubation and not developing papillae.   These colonies do not breed true.

The two colony types gave identical fermentation reactions.

b. Shigella dispar colony.   These strains showed colonies in every way similar to the granular type of the Sonnei colonies— including the formation of small, smooth secondary papillae which became lactose-positive.

c. Shigella alkalescens colony.   These strains showed a somewhat smoother granular type of colony and they also later developed secondary papillae.   These secondary papillae, however, always remained consistently lactose-negative.

d. Shigella paradysenteriae (Flexner's bacillus). Secondary papillae were less frequently encountered in the Flexner type. When they did appear they also remained consistently lactose-negative. The Flexner colony, in general, was the smoothest of all.

2. *Microscopic appearance*

Smears from the various colonies showed approximately the same morphology; all were gram-negative rods. The only difference noted was that the old strains seemed to contain more filamentous forms than freshly isolated strains.

3. *Motility*

Motility was checked on all strains by observing hanging drop preparations on eight- and on eighteen-hour cultures in beef-infusion broth at room temperature and at 37°C. In addition, each strain was tested for migration through semisolid agar at room temperature and at 37°C. All strains were uniformly non-motile.

4. *Biochemical reactions*

The reactions shown in table 1 were found to be the most useful in separating the groups. The sugar media consisted of 1 per cent of sugar in 1 per cent peptone water with Andrade indicator. The tubes were kept at 37°C. for sixty days. At first, two methods of controlling evaporation were used: either keeping the tubes filled to a mark with sterile distilled water, or waxing the cotton stoppers with paraffin. After twenty-three strains had shown identical results by both methods, the waxed plug method was adopted for the rest of the work in order to avoid the occasional contamination.

The litmus milk was kept in a tube with a waxed plug.

Indole was tested for by treating two-day and five-day growths in 1 per cent peptone water with Ehrlich's reagents.

Some of the type strains were put through the full examination only once, but most of the recently isolated strains were re-examined and the tests were repeated for all strains which showed

results not conforming with the general results. In these repeat tests, the only difference found was that two strains which failed to ferment lactose in the first sixty-day trial, did ferment lactose on retest in 27 and 32 days respectively.

As shown in table 1, both *Shigella sonnei* and *Shigella dispar* are sharply distinguished from *Shigella alkalescens* and *Shigella paradysenteriae* (flexner), and, incidentally, from all other *Shigella*, in the late fermentation of lactose. The Sonne group may be

TABLE 1

The most useful reactions for separating the mannitol-fermenting groups of dysentery bacteria

CLASSIFI-CATION	NUMBER OF STRAINS	GLUCOSE	SUCROSE	LACTOSE	MANNITOL	RHAMNOSE	XYLOSE	SORBITOL	DULCITOL	INDOLE	LITMUS MILK
Sonne	54	A	A 4-32 days	A 2-32 days	A	A	0 Very rarely A	0	0	0	Neutral to A about 2 days after Lactose
Dispar	15	A	A 2-19 days	A 1-6 days	A	A	A	A	0	+	Neutral to A about 2 to 8 days
Alkalescens	14	A	0	0	A	A 3-17 days	A	A	A	+	Neutral to very alkaline 3 to 10 days
Flexner	Compiled	A	var.	0	A	var.	0	var.	0	var.	var., A changing to alkaline

All strains non-motile, gelatin negative, salicin negative.

A = acid; 0 = no change during 60 days incubation; var. = variable, depending on the particular strain tested.

separated from the dispar group since the latter ferment xylose and sorbitol and produce indole.

Shigella alkalescens stands distinctly apart from the others in the production of alkalinity in litmus milk, a feature of diagnostic significance, with the fermentation of dulcitol.

The reactions of the Flexner group are not based on the five strains used in this study alone, but compiled from the literature as well as our own experience with other Flexner strains in the past.

In the fermentation of mannitol, all of these groups are related. As Murray (1918), Andrewes and Inman (1919), and Gardner (1929) point out, this is the one important fermentable substance of taxonomic value which has stood the test of time.

It is apparent from the data given in table I that complete identification of *Shigella sonnei* by cultural tests alone cannot be made within a time short enough to be of clinical value. The distinguishing changes in lactose and litmus milk often take two or three weeks to develop. Thus, for rapid diagnosis any mannitol-fermenting dysentery organism must be subjected to serological tests.

B. ANTIGENIC PROPERTIES

The serological properties of the groups were studied by the use of agglutination, agglutinogenic power and the agglutinin absorption reaction.

Methods

From an original plate the smoothest colony was selected and large agar plates inoculated. Incubation was for eighteen hours in order to avoid the occurrence of secondary papillae (lactose-positive variants) which, in our experience, never appeared under forty-eight hours. The growth was scraped from the surface of the plate with a spade and suspended in 0.85 per cent NaCl to which 0.3 per cent of formalin was added. Such suspensions remained stable, having no tendency to sediment spontaneously as "rough" strains would be expected to do. They were standardized by comparison with standard opacity tubes by Brown's method.

Rabbits were immunized by the intravenous injection of 500 million, 1000 million and 2000 million formalized bacteria on the first, sixth and eleventh days respectively. The animals were bled on the seventeenth day and the serum obtained usually reached a titre of between 5,000 and 40,000.

For agglutination tests, the antigen suspensions were standardized to a final concentration of 400 million bacteria in each tube, the total volume of fluid being 2.0 cc. The range of serum

dilutions in each test was from 1 in 40 to 1 in 80,000. Lower dilutions were avoided because experience had shown that normal rabbit serum often agglutinated various dysentery bacteria in lower dilution but that the titre rarely exceeded 1 in 40. The end-point selected in each test was the greatest dilution of serum which gave complete agglutination and a clear supernatant fluid after twenty-four hours incubation at 37°C.

For agglutinin absorption tests the antigens were standardized to a final concentration of 135,000 million bacteria per cc. of undiluted serum. Absorptions were done in a final 1-in-10 dilution of serum in a total volume of 10 cc; incubated for 5 hours at 37°C.; then left in the ice box overnight before centrifuging. The absorbed serum was then tested not only against the homologous strain but also against several others.

At the beginning of this study an agglutinating serum was prepared in rabbits for all of the strains available at that time. Twenty-seven serums in all were prepared against twenty-seven different strains. Of these, 14 were *S. sonnei*, 5 were *S. paradysenteriae* (flexner), 4 were *S. alkalescens* and 4 were *S. dispar*. Later, additional strains were added to the series, so that our results are finally based on tests involving twenty-seven serums and eighty-eight strains. 54 of these strains were finally classified as *S. sonnei*, 14 as *S. alkalescens*, 15 as *S. dispar* and 5 were type strains of *S. paradysenteriae* (flexner).

Table 2 shows the result of cross agglutinations between 14 serums and 22 strains of *S. sonnei*.

The existence of two groups seems indicated by the table; neglecting, for the moment, differences in agglutinability of strains—a factor not controlled in this type of experiment but capable of being checked by absorption tests.

The first group included 13 strains isolated in this laboratory and 3 National Type Collection strains. Nine other National Type Collection strains as well as 17 other strains isolated in this laboratory are not listed in the table but have been shown to fall into this group. Each strain is agglutinated to full titer by a serum produced from any other strain. This result is in agreement with that of many previous investigators who contend that

the sonnei group is antigenically homogeneous, (Kruse (1907), Hilgers (1920), Fraser, Kinlock & Smith (1926), Kerrin (1928), Nelson (1930) and Koser, Reiter, Bortniker and Swingle (1932)),

TABLE 2

Cross agglutinations of 22 strains of Shigella sonnei with rabbit agglutinating serums prepared from 14 of these strains

ANTIGEN	ANTISERUM													
	Nat. type col. 2318	Nat. type col. 2182	Nat. type col. 268	B593	CMH683	CMH218	B314	CMH212	CMH207	CMH232	A3252	B637	B217	B597
Nat. type col. 2318...........	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Nat. type col. 2182...........	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Nat. type col. 268............	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B593.........................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH683.......................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH218.......................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B314.........................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH212.......................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH207.......................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH232.......................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
A3252........................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B637.........................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH8122-b....................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH8187-1b...................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH8187-2a...................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH8222-1b...................	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B217.........................	5	3	2	4	2	3	2	2	2	2	3	2	10	10
B597.........................	4	2	2	4	2	3	2	2	2	2	3	2	10	10
CMH8122-a....................	5	2	3	3	2	3	3	2	2	3	3	2	10	10
CMH8187-1a...................	3	3	2	4	3	2	2	2	2	2	2	2	10	10
CMH8207......................	2	2	2	2	3	2	2	2	2	2	2	2	10	10
CMH8222-1a...................	5	3	2	4	2	3	2	2	2	2	3	2	10	10

Numbers refer to the end point tube (complete agglutination) in a series where 10 represents the titre for the homologous strain.

though others (Sonne (1915), Murakami (1927), Leuchs and Plockmann (1927)) suggest the existence of two serological varieties. Since it is apparently the most common type of the sonnei organism isolated, we propose calling this group *Shigella sonnei*, Type I.

The second group includes 6 strains isolated in this laboratory. An additional 6 strains, also isolated here but not listed in the table, fall into this group as well. These strains are agglutinated to not more than a small fraction of the titre by any serum of Type I. However, a serum prepared from two of these strains, B217 and B597, will agglutinate all strains of both types to full titre. Since this group is less commonly found than the former we propose calling it *Shigella sonnei*, Type II. It should be recorded that this Type II is probably more distinctive of the species *Shigella sonnei* than Type I as judged by taxonomic standards—a point which will be further supported by later evidence.

In order to check these results and to control the factor of variation in agglutinability of strains, absorption tests were carried out.

Table 3 shows the result of some of these absorptions. The table is condensed from a large series of absorptions in which the results illustrated were characteristic of the entire series.

These absorptions confirm the results of straight agglutination tests as to the existence of two types of *Shigella sonnei*. They also warrant additional statements regarding the antigenic structure of the two types.

Type II evidently contains both Type I and Type II antigens in considerable amounts because, not only will its serum agglutinate both types to full titre, but it will itself completely absorb the agglutinins from serum of both types.

Type I, on the other hand, apparently contains the Type I antigen in predominance, with a relatively small amount of Type II antigen, since its serum will agglutinate Type II to only a fraction of its titre, and it will absorb not more than 50 to 75 per cent of the agglutinins from a Type II serum.

The question might arise whether we are dealing here with a smooth-rough type of variation such as Johnston and Kaake (1932) concluded was the explanation of the two types described by Johnston and Brown (1930). In its original definition the smooth-rough variation was related to differences in colony form, kind of growth in broth and salt sensitiveness and more recently

to loss of an antigenic component. Although the Sonne colony is never quite as smooth as the Flexner colony even on primary isolation it grows with uniform turbidity in broth, does not sediment spontaneously in saline and the two colony forms we describe are not related to the immunological types by agglutination.

TABLE 3

Reciprocal absorptions between two Type I strains of Shigella sonnei (nat. type col. 2318 and B593) and two Type II strains (B217 and B597)

	SERUM	ABSORBING STRAIN	ABSORBING DOSE OF UNDILUTED SERUM	TITRE FOR HOMOLOGOUS STRAIN		REDUCTION OF HOMOLOGOUS TITRE
				Unabsorbed	After absorption	
			millions per cc.			*per cent*
Homologous absorptions Type I	N.T.C.2318	B593	135,000	5,120	0	100
	N.T.C.2318	N.T.C.2318	135,000	5,120	0	100
	B593	N.T.C.2318	135,000	40,000	640	99
	B593	B593	135,000	40,000	80	99
Heterologous absorptions	N.T.C.2318	B217	135,000	5,120	0	100
	N.T.C.2318	B597	135,000	5,120	0	100
	B593	B217	135,000	40,000	1,280	96
	B593	B597	135,000	40,000	640	99
	B217	N.T.C.2318	135,000	2,500	1,280	50
	B217	B593	135,000	2,500	1,280	50
	B597	N.T.C.2318	135,000	2,500	640	75
	B597	B593	135,000	2,500	1,280	50
Homologous absorptions Type II	B217	B597	135,000	2,500	0	100
	B597	B217	135,000	2,500	0	100

Referring to the clinical records we find that in some instances different colonies of the same original stool plate fall into different serological types. Thus, in case CMH 8122, colony *a* is Type II while colony *b* is Type I. In case CMH 8187, colony *1a* is Type II while colonies *1b* and *2a* are Type I. In case CMH 8222 colony *1a* is Type II while colony *1b* is Type I. These observations might suggest that we are dealing with a *diphasic* variation which has hitherto been considered to apply only to flagellated species of bacteria. (See however, Takita (1937).)

Our data at present do not permit a decision on these points and to pursue them further would unnecessarily prolong this paper and would deviate from our original purpose.

Tentatively, we suggest that *Shigella sonnei* contains two predominant antigens which we call Type I and Type II. The Type II is the more distinctive Sonne bacillus in that it contains a considerable amount of both antigens while the Type I seems to lack some antigen present in Type II. In other words, an anti-Type II serum will agglutinate all types of Sonne strains that we have encountered but an anti-Type I serum will fail to agglutinate some of them except in fractions of the serum titre.

Having determined the major antigens in *Shigella sonnei* we next studied the minor antigens which relate it to the other mannitol-fermenting groups of dysentery bacteria.

Table 4 is condensed from a larger series to illustrate these relationships.

It is apparent from this table that *Shigella dispar* is itself antigenically heterogeneous; this confirms the observations of Forsyth (1933) and of Watanabe (1935). The Flexner antigens (*Shigella paradysenteriae*) are omitted from the table since it is well known that they are heterogeneous. *Shigella alkalescens* appears to be homogeneous and it may be remarked that 10 additional strains not listed in the table were also agglutinated to full titre by the four Alkalescens serums.

The Sonne Type I antigen is agglutinated to a slight extent by Flexner, Alkalescens and Dispar serums. It is a curious fact that, in a larger series comprising 42 strains of Sonne, every one showed some agglutination with Flexner V, W, X and Z serums but uniformly failed to be agglutinated by a Flexner Y serum. In contrast to this the Alkalescens antigens were uniformly agglutinated by V, W, X and Y serums and failed to be agglutinated by a Flexner Z serum.

The Sonne Type II antigens showed less obvious relation to either Flexner, Alkalescens or Dispar. Of the two antigens illustrated, B597 appears to be the more nearly a pure *Shigella sonnei* antigen in that it fails to react with any of these serums while B217 reacts with both Flexner Z and Alkalescens serums—a property of this strain not disclosed by any of our previous tests.

TABLE 4

Cross agglutination of representative strains of Shigella sonnei Type I and Type II, Shigella alkalescens and Shigella dispar with Flexner, Alkalescens, Dispar and Sonne serums

Antiserum

	ANTIGEN	FLEXNER					ALKALESCENS				DISPAR				BONNE	
		"V" Lister	"W" D.J.	"X" Toner	"Y" Ledingham	"Z" Whittington	Alkalescens "Andrewes"	B598	B599	B1374	Nat. type col. 1602	Nat. type col. 4645	Nat. type col. 4168	"E" Ledingham (Murray class 4)	Nat. type col. 2318 (Type I)	B597 (Type II)
Shigella sonnei Type I	Nat. type col. 2318	2	1	2	0	4	5	4	4	5	3	6	2	2	10	10
	Nat. type col. 2182	2	2	2	0	4	5	4	4	5	2	4	2	2	10	10
	Nat. type col. 268	2	2	2	0	5	5	4	4	5	3	4	2	2	10	10
	B503	3	4	3	0	5	5	4	4	5	3	4	2	2	10	10
	CMH683	2	3	2	0	5	5	4	4	5	2	3	2	2	10	10
	CMH218	2	2	2	0	5	5	4	4	5	3	2	2	2	10	10
Shigella sonnei Type II	B217	0	0	0	0	2	5	2	3	5	0	0	0	0	5	10
	B597	0	0	0	0	0	0	0	0	0	0	0	0	0	4	10
Shigella alkalescens	Alkalescens "Andrewes"	6	4	4	4	0	10	10	10	10	6	5	0	0	0	0
	B598	6	5	4	5	0	10	10	10	10	5	5	0	0	0	0
	B599	6	5	4	5	0	10	10	10	10	6	4	0	0	0	0
	B1374	3	3	4	4	0	10	10	10	10	5	4	0	0	0	0
Shigella dispar	Nat. type col. 1602			3	2		0				10	0	10	10	0	3
	Nat. type col. 4645			4	2		2				0	10	0	0	0	0
	Nat. type col. 4168			4	3		0				10	8	10	10	0	2
	"E" Ledingham (Murray class 4)			4	2		5				10	0	10	10	0	0
	19A (Murray class 4)			4	2		2				10	0	10	10	0	0
	67B (Murray class 4)			4	2		3				10	0	10	10	0	0
	S6A (Murray class 1)			4	2		2				0	0	10	10	0	0
	A3031			4	2		1				10	10	0	0	0	0

Numbers refer to the end point tube (complete agglutination) in a series where 10 represents the titre for the homologous strain. Blank spaces mean that the test was not carried out.

The results of table 4 again confirm our views that the Type II Sonne bacillus is the more typical of the species in that its antigens are more redominantly *Shigella sonnei* in character. If it contains the antigens common to the Flexner, Alkalescens and Dispar groups, these are not brought into evidence by this type of test.

Reciprocal absorption tests failed to add to the information given in table 4.

DISCUSSION

If the dysentery group as a whole be divided primarily on a basis of mannitol fermentation we find that the non-mannitol-fermenters, *Shigella dysenteriae*, *Shigella ambigua*, (Schmitz bacillus) stand sharply apart from the others by reason of a number of properties; cultural, antigenic and pathogenic. The next subdivision, among the mannitol fermenters themselves, is not so clear cut and overlapping occurs in many of the properties of each species. The most striking and most reliable feature which can be used to separate the Sonnei-Dispar group from the Flexner group is the fermentation of lactose, but this may be delayed as long as three or four weeks. Because the individual strains of the Flexner group are so variable in themselves it is impossible to distinguish between Flexner and Sonne or Dispar on cultural grounds alone until sufficient time has been allowed for the fermentation of lactose. Serologically also, enough overlapping occurs to require a complete series of agglutinations to the full titer of the serum before reliance can be placed on this type of information.

It is, of course, just this similarity of properties among the mannitol-fermenting dysentery organisms that justifies considering them more closely related to one another than is the relationship of any one of them to either Shiga or Schmitz.

We believe that Alkalescens belongs in the mannitol-fermenting group of dysentery organisms in spite of its indefinite pathogenicity. From a taxonomic viewpoint, pathogenicity must be regarded as only one of the properties of a species, not necessarily more important *per se* than a combination of other proper-

ties. For similar reasons we have excluded from our study such species as have been described under the name of "Coli Anaerogenes." On closer examination some of these strains are either true Sonne or Dispar or they produce small quantities of gas, often late, and thus appear to be more nearly related to the Escherichiae (Coli-Aerogenes group) than to the dysentery group. (Topley and Wilson 1936)).

The occurrence of secondary papillae on colonies is not confined to members of the Genus *Shigella*; it is occasionally found also among other genera in *Eberthella* and *Salmonella*. But in the Genus *Shigella* it reaches a marked development. Less commonly seen in the Shiga and Schmitz species, it becomes more frequent in the Flexner and Alkalescens species and is the rule rather than the exception in Sonne and Dispar. So much is this so, that the phenomenon of late lactose fermentation in Sonne and Dispar seems dependent on the production of these papillae. They are, in fact, variants of the original strains with new fermenting powers. We have consistently failed to get the variants to breed true; they also revert to the lactose-negative state on fresh media and the fermentation of this sugar occurs only in ageing cultures. The failure to secure a pure culture of lactose-positive Sonne has prevented our using it for antigenic analysis.

While we feel certain of the presence of at least two major antigens in *Shigella sonnei*, we fine no indication that they are associated with a rough-smooth variation. If "roughness" be defined as a rough colony which does not remain in suspension in the presence of electrolytes, these antigens are both smooth antigens. If, on the other hand, "roughness" is defined as the quantitative loss of some antigenic component, we find that our Type II possesses an antigenic component not present in our Type I; but both serological types are independent of the variation observed in colony form. We have not investigated the possibility of a diphasic variation.

From the standpoint of practical diagnosis only the Type II antibody can be relied on to detect all strains of *Shigella sonnei*; the Type II antibody will agglutinate all strains to full titre while the Type I antibody will agglutinate only the Type I

antigen to full titre and the type II antigen to a fraction of its titer.

The minor antigens of *Shigella sonnei* which show cross reactions with *Shigella paradysenteriae, Shigella alkalescens* and *Shigella dispar* again emphasize the close taxonomic relationship of these four species. It is interesting that Type II, which is regarded as the more distinctive, shows fewer cross reactions with the other three than does Type I.

We have searched for, and failed to find, any cultural or biochemical property by which Type I strains might be distinguished from Type II strains. All of our Type II strains have been recent isolations but an even greater number of recent strains belong to Type I. Indeed, both types have occurred at the same time on the same original plate from the same stool specimen.

SUMMARY AND CONCLUSIONS

1. *Shigella sonnei* is closely related culturally and antigenically to three other members of the mannitol-fermenting species of dysentery organisms; namely the *Shigella paradysenteriae* (Flexner's bacillus), *Shigella alkalescens* and *Shigella dispar*.

2. The most outstanding distinguishing property of *Shigella sonnei*, the late fermentation of lactose, is due to the appearance in ageing cultures of variants with new fermenting powers. These variants are seen in the original colony as secondary papillae arising directly from the mother colony only after the colony has aged.

3. These variants are not permanent. When transferred to fresh medium, they immediately revert to the lactose-negative state and, eventually, these transfers in turn produce lactose positive variants. This succession of events may be continued indefinitely.

4. At least two major antigens are present in *Shigella sonnei* both of which are present in all strains but to varying degrees. *Shigella sonnei* Type II is the more representative type since both antigens are abundantly represented in it; but it is least frequently encountered. *Shigella sonnei* Type I is most fre-

quently found and contains mainly Type I antigen with a relatively small amount of Type II antigen.

5. Minor antigens in *Shigella sonnei* are common to *Shigella dispar*, *Shigella alkalescens*, *Shigella paradysenteriae*.

6. On a basis of cultural and antigenic properties the Sonne group differs sufficiently from the closely related Flexner, Dispar and Alkalescens groups to warrant recognition as a separate species of the Genus *Shigella* rather than as a variant of *Shigella paradysenteriae*. This confirms the species *Shigella sonnei* (Weldin).

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THE ABSORPTION OF BACTERIOPHAGE BY SENSITIZED ENTEROCOCCI

ELBERTON J. TIFFANY AND MORRIS L. RAKIETEN

Department of Bacteriology, Long Island College of Medicine, Brooklyn, N. Y.

It is generally accepted that the first step that takes place in the lysis of bacteria by a bacteriophage consists in the absorption of the bacteriophage particle to the surface of the organism, and that organisms that are phage-resistant will not absorb bacteriophage. Exceptions to this principle, namely where a non-susceptible organism is capable of binding phage to a high degree, have been encountered by Levine, Frisch, and Cohen (1934), Burnet (1934) ,Rakieten *et al.* (1936), and Rakieten and Tiffany (1938). This preliminary absorption by the bacterial cell is in all probability due to some element of the complex antigenic structure of the organism. Burnet, in a series of investigations dealing with bacteria belonging to the dysentery and salmonella groups and their appropriate phages, has shown that a marked parallelism exists between antigenic reactions and phage susceptibility, and has brought forth evidence pointing to the heat-stable somatic or O-antigen as the fraction which specifically absorbs phage. *Salmonella* strains having a minor O-antigen in common with *Salmonella enteritidis* showed a degree of sensitivity to some of the enteritidis phages, whereas this sensitivity was, with one exception, lacking among organisms not possessing an O-antigen in common with *S. enteritidis* (Burnet, 1927; Burnet and McKie, 1930; Burnet, 1930, 1932, 1934; Gough and Burnet, 1934). Thus, the evidence that the phage-binding portion of the bacterial cell is linked with that portion of the cell that binds the O-agglutinin appears to be highly convincing.

If the component of the bacterial cell capable of binding phage is the agglutinogen, then, when this antigen is united with spe-

cific agglutinin, phage absorption by these cells should be negligible, as compared with the degree of positive absorption by bacterial cells whose heat-stable surface antigen has been untouched by specific sera.   In order to test this hypothesis a number of experiments were carried out.   The results that were obtained confirmed the above hypothesis, namely that there is a direct relation between "covering" of agglutinogen and lack of absorption of phage.   At the same time numerous attempts were made to prove that the agglomeration of these cells was not in itself the reason for their failure to fix phage.   The present report deals with the experimental data obtained to substantiate our conclusions.

The organisms used were three members of the enterococcus group, strains Z-1, Z-11, and E-16.   Cultures Z-1 and Z-11 belong to the species *Streptococcus liquefaciens* according to Sherman's classification (Sherman, 1937), and had originally been isolated from the blood of patients with sub-acute bacterial endocarditis.   Strain E-16 was isolated from a case of ulcerative colitis, and was typical of *Streptococcus fecalis*.   These organisms, together with other members of the group have been used in a previous investigation and are described more completely in a communication recently published (Rakieten and Tiffany, 1938).

The sera were prepared by repeated intravenous inoculation in rabbits of formalin-killed vaccines of the appropriate organisms and were preserved in 1:10,000 merthiolate.   Serum 84 was prepared against culture Z-1; serum 11 against culture Z-11; and serum 87 against strain E-16.   All of these cultures were related closely serologically, but were not identical as may be seen by observing tables 1a and 1b, in which the agglutination and agglutinin absorption titers are presented.

In table 1a the figures represent the highest dilution at which agglutination was unequivocal, although not necessarily complete.   The italic figures represent the titers obtained with homologous systems.   In table 1b the figures showing the titer of serum with homologous organism after absorption by an heterologous organism are italic.   From this last table it is clear that there is no complete reciprocal absorption and that no one

of the three organisms is serologically identical with another, although they do have some degree of antigenic similarity. The only system in which there is important absorption of the homologous or "major" agglutinin of a serum by a heterologous organism is that in which serum 11 is absorbed with culture E-16. Strain Z-11 on the other hand leaves untouched the "major" agglutinin in serum 87. Thus, these two organisms, Z-11 and E-16, while not identical, are more closely related to one another than is either to culture Z-1. Such one-sided relationships be-

TABLE 1a

Serum titers for homologous and heterologous organisms before absorption

	SERUM 84 (Z-1)	SERUM 11 (Z-11)	SERUM 87 (E-16)
Tested vs. strain Z-1....................	*5000*	2560	2560
Tested vs. strain Z-11...................	2560	*2560*	5000
Tested vs. strain E-16..................	2560	1280	*5000*

TABLE 1b

Relationship of strains as shown by reciprocal absorptions

	SERUM 84 (Z-1)	SERUM 11 (Z-11)	SERUM 84 (Z-1)	SERUM 87 (E-16)	SERUM 11 (Z-11)	SERUM 87 (E-16)
	Absorbed with culture					
	Z-11	Z-1	E-16	Z-1	E-16	Z-11
Tested vs. strain Z-1......	*5000*	80	*1280*	<20		
Tested vs. strain Z-11.....	80	*1280*			<40	320
Tested vs. strain E-16.....			160	*5000*	80	*5000*

tween organisms are known, and are described in some detail by Krumwiede, Cooper, and Provost (1925). These authors state that "between the two degrees of a complete reciprocal reaction and an absence of such reaction, all degrees of partial reciprocal reactions may be encountered." None of these sera contained antibodies specific for Lancefield's Group D streptococcus.

The bacteriophage employed is polyvalent for many strains of enterococci and was propagated against these cultures many times in Savita broth (Rakieten, 1932). This phage is com-

pletely absorbed by heat-killed cultures of Z-1, Z-11, and E-16, as well as by watery extracts prepared from these same strains. Unless stated otherwise, the suspending medium used in this study was Savita broth.

EXPERIMENTAL

The method used to demonstrate the blocking effect of immune antibodies upon the so-called phage receptors of the bacterial cells was as follows. Standard suspensions of cultures which had been killed by exposure in sealed ampoules to a temperature of 70°C. for thirty minutes, were suspended in broth containing known dilutions of anti-serum. After an incubation period of from one to three hours in a water bath at 37°C., the degree of agglutination was determined microscopically; following this, a constant volume of bacteriophage was added to each tube and the entire series incubated for twenty hours at 35°C. The amount of bacteriophage added to each tube was such that, providing no absorption took place, a countable number of plaques was produced. 0.02 cc. from each tube was then layered over a segment of an agar plate that had previously been inoculated with a fresh susceptible culture of Z-1. Throughout this work culture Z-1 has been used to demonstrate plaque production, since it was found that the results were approximately the same regardless of which organism was employed as the indicator. The tubes were again checked for evidence of agglutination by the use of the microscope. Plaque counts were done after an appropriate incubation of the agar plates. The degree of agglutination was thus known and recorded by one of us long before the plaque counts were made by the other. Throughout the entire study the individual responsible for adding phage and carrying the experiment to completion did not know the contents of the tubes containing the various mixtures. Proper controls were always run with each new series of experiments.

Experiment 1. The phage-absorbing power of a heavy suspension of organisms in the presence of homologous anti-serum

Heat killed suspensions of culture Z-1 in broth were set up in a series of tubes containing different dilutions of homologous im-

mune serum as well as normal rabbit serum. The total volume in each tube was 5 cc., the concentration of organisms was four billion per cubic centimeter. After a preliminary incubation period of three hours, a constant quantity of bacteriophage was added to each tube. Following an incubation period of twenty hours, the tubes were examined for agglutination and portions removed and tested for their ability to produce plaques. Table 2 illustrates the results obtained.

The results set forth in this table indicate that the antibodies present, while capable of agglutinating the bacteria, did not,

TABLE 2

TUBE NUMBER	ORGANISM SUSPENSION	SERUM DILUTION (BP. ADDED)	AGGLUTI-NATION	PLAQUE COUNT
1	Heat killed Z-1	IS 84, 1:50	4+	0
2	Heat killed Z-1	IS 84, 1:160	4+	0
3	Heat killed Z-1	IS 84, 1:2000	±	0
4	Heat killed Z-1	NRS, 1:50	−	0
5	Heat killed Z-1	NRS, 1:160	−	0
6	Heat killed Z-1	NRS, 1:2000	−	0
7	None	IS 84, 1:50		cfl
8	None	IS 84, 1:160		cfl
9	None	IS 84, 1:2000		cfl
10	Heat killed Z-1	None		0
11	None	Savita broth. None		cfl

IS = immune rabbit serum; NRS = normal rabbit serum; cfl = plaques confluent (over 500); 4+ = complete agglutination, macroscopically, but microscopic examination revealed many unagglutinated organisms.

under the conditions of this experiment, in any way interfere with the complete absorption of the added bacteriophage. In this heavy suspension, however, microscopic examination revealed free and unagglutinated cells in such quantitities that, conceivably in the absence of complete coating by the antibodies, they alone could be responsible for the fixation of the bacteriophage. It is of interest to note that in no instance did any of the dilutions of serum inhibit the phage. These findings, which were obtained many times in repeating similar experiments, suggest that any interference with phage absorption by surface antigen in the presence of immune serum might be demonstrable only after a careful adjustment of the various elements employed in the test.

Experiment 2. The titration of reagents

An effort was made to determine how far one could dilute the standard suspension of bacteria and still get significant absorption of the added phage. A heat-killed suspension of culture Z-1 (four billion per cubic centimeter) was prepared in the usual manner and serial dilutions set up in broth, the final volume being 5 cc. Constant quantities of diluted bacteriophage were then added and the tubes incubated for twenty hours at 35°C. 0.02 cc. from each tube was then layered over a segment of an agar plate that had previously been streaked with culture Z-1.

TABLE 3

TUBE NUMBER	SUSPENSION (HEAT-KILLED Z-1) 4 BILLION PER CC., PLUS PHAGE	PLAQUE COUNT
1	Undiluted	0
2	*Diluted 1:2*	*0*
3	Diluted 1:4	0
4	Diluted 1:8	0
5	Diluted 1:16	0
6	Diluted 1:32	0
7	Diluted 1:64	0
8	Diluted 1:128	0
9	Diluted 1:256	4
10	Diluted 1:512	13
11	Savita broth	500

After incubation the plates were examined for the presence of plaques and counted. The results are listed in table 3.

The turbidity of the suspension decreased uniformly to tubes 8 and 9, in which cloudiness was barely perceptible. Tube 10 in any light appeared as clear as the control without organisms (tube 11). Tubes 8, 9 and 10, when examined microscopically by hanging drop and smear, showed very few organisms (the calculated number of organisms in tube 10 is less than 8,000,000 per cubic centimeter). These results suggested that in the previous experiment (table 2) suspensions far too heavy may have been employed, since a suspension having the same density and diluted 512 times is still capable of inactivating phage.

Therefore, in the second part of this experiment a standard

suspension of heat-killed culture of Z-1 of nearly the same density (3.8 billion per cubic centimeter) was made and serial dilutions prepared. To each dilution a constant amount of immune and normal rabbit serum was added, and, following an hours incubation, 0.1 cc. of bacteriophage was inoculated into each tube. After remaining at 35°C. for twenty hours a portion from each

TABLE 4

TUBE NUMBER	SUSPENSION (HEAT-KILLED Z-1) 3.8 BILLION PER CC.	SERUM DILUTION (BP. ADDED)	AGGLUTINA- TION	PLAQUE COUNT
1	Undiluted	IS 84, 1:50	+	0
2	Undiluted	NRS, 1:50	±	0
3	Undiluted*		−	0
4	Diluted 1:4	IS 84, 1:50	+	1
5	Diluted 1:4	NRS, 1:50	−	0
6	Diluted 1:4*		−	0
7	Diluted 1:16	IS 84, 1:50	+	43
8	Diluted 1:16	NRS, 1:50	−	0
9	Diluted 1:16*		−	9
10	Diluted 1:64	IS 84, 1:50	±	296
11	Diluted 1:64	NRS, 1:50	−	6
12	Diluted 1:64*		−	0
13	Diluted 1:128	IS 84, 1:50	−	314
14	Diluted 1:128	NRS, 1:50	−	43
15	Diluted 1:128*		−	0
16	Diluted 1:512	IS 84, 1:50	−	380
17	Diluted 1:512	NRS, 1:50	−	380
18	Diluted 1:512*		−	5
19	None*	IS 84, 1:50		379
20	None*	NRS, 1:50		450
21	None*	None		450

* Broth.

tube was removed (0.02 cc.) and tested for its ability to produce plaques. The results are indicated in table 4.

With a heavy suspension of organisms (undiluted, to a dilution of 1:16) the immune serum in the dilution used is unable to affect demonstrably the phage-absorbing power of these cells. When the number of bacteria is progressively decreased, and these cells are allowed to come in contact with the same dilution of immune serum, the surface antigens of these cells do appear to

be completely "covered" by the antibodies, for when phage is added to these cells there is no significant reduction in corpuscular count.   It is important to note that while, in general, the normal rabbit serum has little or no effect on these same cell receptors and therefore phage is absorbed, that in certain of the bacterial dilutions normal rabbit serum does seem to be able to cover surface antigen so that it is not free to absorb bacteriophage, (tubes 14 and 17).   The normal rabbit serum used showed a definite agglutination of culture Z-1 when the serum was diluted 1:40, and a slight amount of agglutination in a dilution of 1:80. It is quite likely, therefore, with the small number of organisms present in tube 17, that the antibody concentration in the 1:50 dilution of normal serum was not without effect, and combined with the surface antigen to such an extent that phage was not absorbed.   Again, it may be observed that normal or immune serum alone, in the dilution used, has no effect on the phage.   In tubes 1 and 4, where large numbers of organisms were present, the agglutinating effect of the serum was marked.   In the 1:16 dilution of bacteria the clumps of organisms were very small, consisting of two or three pairs of cocci, but the great majority of bacteria were not agglutinated.   Agglutination was doubtful in tube 10 (dilution 1:64), and in the dilutions of 1:128, and 1:512 there were 10–12 organisms per high power field with no clumps observed.   These observations were made after one hours incubation at 37°C.

The results shown in table 4 may be explained as follows: (1) either the antibodies absorbed to the bacterial surface make any contact impossible between the bacteriophage and the bacterial phage receptors, in other words phage absorption is blocked, or (2) the aggregation of organisms in clumps so reduces the chance of contact between phage particles and bacteria that very little is absorbed, leaving a large amount free to produce plaques when placed in contact with susceptible bacteria.

We have given considerable attention to the possible rôle of agglutination as being responsible for our results, and after several experiments were done in which organisms agglutinated by lanthanum chloride were found to absorb phage as completely

as the unagglutinated controls, we feel certain that the reduced phage-absorbing capacity of suspensions of sensitized heat-killed organisms is due to a "coating" of surface antigen. Adequate evidence that our results are independent of mere mechanical clumping is shown in subsequent tables, where almost complete absorption of bacteriophage occurs in the presence of marked agglutination.

Experiment 3. The effect of varying dilutions of antiserum on the phage-absorbing power of a constant quantity of organisms

The suspending fluid was a peptone hypotonic saline solution, containing 0.1 per cent peptone (Parke-Davis) and 0.1 per cent NaCl. The solution was adjusted to pH 7.6 and sterilized in the autoclave. A heat-killed standard suspension of culture Z-1, diluted 1:32 was distributed in tubes and to these were added serial dilutions of homologous immune and normal rabbit serum, making a final dilution of organisms 1:64, and the indicated dilutions of serum. The tubes were incubated at 37°C. for 3 hours, and kept in the ice box overnight before the phage was added. An additional incubation for 20 hours at 35°C. was given, for the phage to come in contact with bacterial surface, before portions were removed from each tube and layered for plaque counts. Examinations for agglutination were made after the first incubation and again just before the phage was added. The results are listed in table 5.

One may observe in table 5 that homologous antibody in a dilution of at least 1:80 is able to coat efficiently the surface antigen so that when phage is added to this sensitized cell suspension a significant number of phage corpuscles are left free and unabsorbed. Bacterial cells treated with normal rabbit serum, on the contrary, are able to absorb the added phage completely. One may also note the lack of parallelism between the amount of agglutination and the number of phage corpuscles left unabsorbed.

The experiments described so far have been concerned with the effect of immune serum on the phage-absorbing power of suspensions of homologous organisms. The following experi-

ments deal with homologous and heterologous organisms and sera. Standard suspensions of cultures of Z-1, Z-11, and E-16 were prepared in Savita broth and killed by heating at 70°C. for 30 minutes. These standards were kept sealed in ampoules in the ice-box and appropriate dilutions made as needed, after washing once with broth. Sterility controls were always done. The organisms in these standard suspensions have retained their Gram-positiveness for the duration of this study, with little if any evidence of autolysis.

TABLE 5

TUBE NUMBER	SUSPENSION (HEAT-KILLED Z-1) 4 BILLION PER CC.	SERUM DILUTION	AGGLUTINATION		PLAQUE COUNT
			3 hrs.	18 hrs.	
1	Diluted 1:64	IS 84, 1:40	±	±	217
2	Diluted 1:64	IS 84, 1:80	±	2+	203
3	Diluted 1:64	IS 84, 1:640	2+	3+	10
4	Diluted 1:64	IS 84, 1:2560	2+	2+	4
5	Diluted 1:64	IS 84, 1:5000	±	±	2
6	None	IS 84, 1:40			230
7	None	IS 84, 1:640			304
8	Diluted 1:64	NRS, 1:40	1+	1+	2
9	Diluted 1:64	NRS, 1:80	±	2+	0
10	Diluted 1:64	NRS, 1:160	±	±	0
11	None	NRS, 1:40			230
12	None	NRS, 1:80			297
13	Diluted 1:64	None			0
14	None	Peptone-saline. None			310

IS = immune serum; NRS = normal rabbit serum.
Each tube contained a constant quantity of bacteriophage.

Experiment 4. The effect of various dilutions of homologous and heterologous sera upon the phage-binding power of a standard suspension of culture Z-11

A constant dilution of this standard suspension was used throughout; all other details with regard to technique follow those described in the previous experiments. The results are set forth in table 6.

This experiment was repeated with standard suspensions of cultures E-16 and Z-1. The results are summarized in table 7.

Here the figures represent the highest dilution of serum in the presence of which 75 per cent or more of the control number of

TABLE 6

TUBE NUMBER	SUSPENSION (HEAT KILLED) CULTURE Z-11	SERUM DILUTION (PLUS BP.)	AGGLUTI-NATION 20 HOURS	PLAQUE COUNT
1	Dilution 1:64	IS 11, 1:10	4+	300
2	Dilution 1:64	IS 11, 1:40	3+	297
3	Dilution 1:64	IS 11, 1:80	3+	143
4	Dilution 1:64	IS 11, 1:320	2+	30
5	Dilution 1:64	IS 11, 1:640	1+	12
6	Dilution 1:64	IS 11, 1:1280	1+	11
7	Dilution 1:64	IS 11, 1:2560	1+	10
8	Dilution 1:64	IS 87, 1:10	2+	300
9	Dilution 1:64	IS 87, 1:40	2+	300
10	Dilution 1:64	IS 87, 1:80	3+	300
11	Dilution 1:64	IS 87, 1:320	3+	197
12	Dilution 1:64	IS 87, 1:640	2+	137
13	Dilution 1:64	IS 87, 1:1280	1+	41
14	Dilution 1:64	IS 87, 1:2560	1+	15
15	Dilution 1:64	IS 84, 1:10	±	140 (cont)
16	Dilution 1:64	IS 84, 1:40	1+	230
17	Dilution 1:64	IS 84, 1:80	2+	141
18	Dilution 1:64	IS 84, 1:320	1+	16
19	Dilution 1:64	IS 84, 1:640	1+	6
20	Dilution 1:64	IS 84, 1:1280	1+	9
21	Dilution 1:64	IS 84, 1:2560	1+	7
22	Dilution 1:64	None		0
23	None	Savita broth		300

IS = immune serum; cont = contaminated.

This series also included control tubes containing serial dilutions of each serum without organisms. In each instance the numbers of plaques obtained approximated the controls.

TABLE 7

The blocking effect of immune serum on homologous and heterologous organisms

STANDARD SUSPENSION DILUTED 1:64	SERUM 84 (Z-1)	SERUM 11 (Z-11)	SERUM 87 (E-16)
Z-1	40	40	10
Z-11	40	40	80
E-16	10	40	80

phage corpuscles failed to be absorbed by the diluted standard suspension.

Upon comparison with table 1, these figures show that, as one would expect, the ability of a serum to coat the surface of an organism, thereby interfering with the absorption of phage, is in keeping with its antibody content for that organism. It is striking that although the maximum agglutinating titer of each serum is far higher, the titer for adequate blockade in the presence of a 1:64 dilution of the standard suspensions that were used is in most cases 1:40, or 1:80. We would expect that with a more dilute suspension of bacteria, a higher dilution of serum should be adequate to coat the surface antigens and therefore prevent the absorption of bacteriophage. The figures shown in table 8, where the organism suspension consisted of a standard suspension of culture E-16 diluted 1:256 confirms this.

TABLE 8

STANDARD SUSPENSION DILUTED 1:256	SERUM 84 (Z-1)	SERUM 11 (Z-11)	SERUM 87 (E-16)
Culture E-16..................	320	80	1280

As in table 7, the figures represent the highest titer of serum in the presence of which 75 per cent or more of the control number of phage corpuscles (as represented by plaque count) are left unabsorbed by the suspension. The difference between the sera is considerably greater in this case and the result with serum 11 is quite out of keeping with what one would expect, as the serological reactions of these organisms as given in table 1 show culture Z-11 to be more closely related to culture E-16 than to culture Z-1. However, one may observe that with a higher dilution of bacteria a considerably less amount of homologous antiserum efficiently coats the surface antigen and renders it non-susceptible to phage.

Experiment 5. The phage-blocking effect of serum from which the minor agglutinins have been removed by absorption

Standard suspensions of heat-killed cultures of Z-1 and Z-11 diluted 1:128 were placed in contact with immune serum 11 (homologous for culture Z-11), from which the group agglutinins

for culture Z-1 had been removed by absorption.   The incubation periods, the examinations for agglutination, and the platings for plaque counts were made as in the previous experiments. The results are presented in table 9.

Reference to tables 1a and 7 respectively, shows that unabsorbed serum 11 agglutinates cultures Z-1 and Z-11 to equal

TABLE 9

TUBE NUMBER	HEAT-KILLED SUSPENSION	SERUM (ABSD.) DILUTION (BP. ADDED)	AGGLUTINATION		PLAQUE COUNT
			3 hrs.	20 hrs.	
1	cult. Z-1, 1:128	IS 11, 1:40	—	—	0
2	cult. Z-1, 1:128	IS 11, 1:80	—	—	2
3	cult. Z-1, 1:128	IS 11, 1:160	—	—	2
4	cult. Z-1, 1:128	IS 11, 1:320	—	—	1
5	cult. Z-1, 1:128	IS 11, 1:640	—	—	0
6	cult. Z-1, 1:128	IS 11, 1:1280	—	—	0
7	cult. Z-1, 1:128	IS 11, 1:2560	—	—	2
8	cult. Z-1, 1:128	IS 11, 1:5120	—	—	1
9	cult. Z-11, 1:128	IS 11, 1:40	—	1+	257
10	cult. Z-11, 1:128	IS 11, 1:80	—	2+	116
11	cult. Z-11, 1:128	IS 11, 1:160	—	3+	61
12	cult. Z-11, 1:128	IS 11, 1:320	—	3+	57
13	cult. Z-11, 1:128	IS 11, 1:640	—	3+	20
14	cult. Z-11, 1:128	IS 11, 1:1280	—	2+	12
15	cult. Z-11, 1:128	IS 11, 1:2560	—	2+	8
16	cult. Z-11, 1:128	IS 11, 1:5120	—	1+	6
17	None	IS 11, 1:40			300
18	None	IS 11, 1:80			300
19	None	IS 11, 1:160			300
20	cult. Z-1, 1:128	None			1
21	cult. Z-11, 1:128	None			0
22	None	Savita broth. None			300

titer and that this serum is as effective in blocking phage absorption by culture Z-1 as by the homologous organism.   The present experiment demonstrates that the removal of group antibodies for culture Z-1 from this serum makes it powerless to affect phage absorption by culture Z-1.   The same serum, however, still retains its ability to combine with the homologous organism, Z-11, and prevents phage absorption.   Again one may

note the lack of parallelism between the degree of agglutination and phage blockade.

According to Burnet (1934) and Rakieten *et al* (1936), when watery extracts of organisms which have a phage-inactivating ability ("PIA") are placed in association with homologous immune serum and the resulting precipitate removed, the supernatants no longer possess this phage-inactivating ability. In the following experiment we investigated the phage-absorbing power of such a watery extract prepared from one of the strains of enterococci before and after the extract had been placed in contact with immune serum.

Experiment 6. The effect of immune serum upon the phage-absorbing capacity of a watery extract of enterococcus

A watery extract from culture Z-1 was made and placed in contact with immune serum 84. An initial dilution of serum of 1:5 was prepared in saline. One cubic centimeter of extract was added to one cubic centimeter of each serum dilution, incubated at 44°C. for one hour, and kept in the ice chest overnight. A definite prozone occurred in the higher concentrations of serum, and in a dilution of 1:80 a visible precipitate resulted. Each tube was centrifuged for 15 minutes at high speed and 1.75 cc. removed without disturbing any of the sediment. These constituted the so-called "supernatants." The same amount of saline was then added to each of the so-called "sediments." To all of the tubes in the series 0.05 cc. of diluted bacteriophage was added and after incubation for 22 hours at 35°C., 0.02 cc. from each tube was layered over susceptible culture Z-1. The plates were examined after the usual 20-hour incubation period and plaques counted. Table 10 summarizes the results.

The plaque counts obtained from tubes 1 to 8 were estimated, all the others were counted. It may be observed from an inspection of the results listed in table 10 that dilutions of serum up to 1:80 are able to remove all the "PIA" from the supernatant and at the same time so coat the precipitinogen with antibody that no absorption of phage takes place. In dilutions of serum above 1:80 the sediments absorb more and more phage, i.e.,

are less completely blocked. These dilutions of serum have been used repeatedly and have never in themselves, exerted any demonstrable effect on the phage. The findings with this and other extracts correspond with those obtained when suspensions of organisms were used.

During the course of this investigation, in several experiments distilled water was used as the medium, but we found that marked reduction in plaque count occurred, even in the absence

TABLE 10

TUBE NUMBER	WATERY EXTRACT	SERUM DILUTION	SUPERNATANT OR SEDIMENT (PLUS BP.)	PLAQUE COUNT
1	culture Z-1	IS 84, 1:10	Supernatant	300
2	culture Z-1	IS 84, 1:10	Sediment	300
3	culture Z-1	IS 84, 1:20	Supernatant	300
4	culture Z-1	IS 84, 1:20	Sediment	300
5	culture Z-1	IS 84, 1:40	Supernatant	300
6	culture Z-1	IS 84, 1:40	Sediment	300
7	culture Z-1	IS 84, 1:80	Supernatant	300
8	culture Z-1	IS 84, 1:80	Sediment	69
9	culture Z-1	IS 84, 1:160	Supernatant	145
10	culture Z-1	IS 84, 1:160	Sediment	87
11	culture Z-1	IS 84, 1:320	Supernatant	184
12	culture Z-1	IS 84, 1:320	Sediment	51
13	culture Z-1	IS 84, 1:640	Supernatant	118
14	culture Z-1	IS 84, 1:640	Sediment	31
15	culture Z-1	IS 84, 1:1280	Supernatant	182
16	culture Z-1	IS 84, 1:1280	Sediment	60
17	culture Z-1	IS 84, 1:2560	Supernatant	71
18	culture Z-1	IS 84, 1:2560	Sediment	12
19	culture Z-1	None	Supernatant	8
20	culture Z-1	None	Sediment	0
21	None	Saline. None	None	300

of bacteria; also that serum diluted in distilled water had an equally deleterious effect. Dilutions of the same serum in Savita broth had no effect on the same phage. In other experiments physiological saline was used as the diluent, and in these instances an even greater destruction of phage was observed. However, the addition of a small quantity of broth or serum (diluted 1:160) "protected" the phage and little or no diminution was observed. These findings appear to be at variance with the

reported experience of Rakieten, Rakieten, and Doff (1936) who state that "...bacteriophage controls were undiminished in titer for as long as 11 days in distilled water, hypertonic or hypotonic salt solution." But under the conditions of their experiments the phages were prepared in Savita broth, and this was added to tubes of distilled water or saline. Hence, a sufficient amount of "protective substance" was present. In the present study the initial dilution was prepared by adding a small loopful (less than 2 mm. in diameter) of phage to 10 cc. of saline. Further dilutions were then made by adding 0.1 cc. of this phage to tubes containing organisms suspended in saline or distilled water, with the result that not enough of the broth was carried over and the phage was destroyed. Reports on the toxicity of saline for other viruses have been made by Nicolau and Galloway (1930) and Goodpasture and Buddingh (1936).

Finally we observed that susceptible organisms suspended in distilled water had little phage-absorbing power. However, the addition of a small amount of peptone (0.1 per cent), and a small amount of electrolyte (0.1 per cent NaCl) to such a suspension in distilled water restored the phage-binding power of the suspension.

DISCUSSION

It has been shown by means of properly adjusted quantities of bacterial cells and immune serum, that the bacterial surface may be coated to such an extent that phage absorption is prevented. On the basis of the results obtained it is believed that the portion of the bacterial cell that specifically absorbs phage is the same as that which absorbs antibody from specific antisera. Since all of the organisms that we have investigated were heat-killed suspensions, it appears that the part of the cell that is primarily responsible for the absorption is the heat-stable agglutinogen. These experiments, however, do not allow us to draw any conclusions with regard to the chemical nature of the antigens involved.

In accord with the reports of Burnet (1934) and Rakieten et al (1936) on watery extracts of bacteria, we have found that

proper concentrations of immune serum are capable of removing all phage-inhibiting power from watery extracts of enterococci. In addition, the precipitate that is produced under conditions where precipitin and precipitinogen are present in optimal proportions, is likewise devoid of phage-inhibiting power. The more direct method of determining the fraction of the cell to which phage attaches itself, chemical analysis, has been tried (Gough and Burnet, 1934) but has not been entirely satisfactory, probably because of marked alterations in the antigens which occur when bacteria are subjected to treatment with chemicals.

CONCLUSIONS

1. The surface component of bacteria to which phage is attached preliminary to lysis can be specifically coated by anti-bacterial immune serum, and by this method rendered inaccessible to phage.

2. The heat-stable agglutinogen of the organisms studied is the surface antigen to which bacteriophage is absorbed.

3. Distilled water or saline alone, under certain conditions, appears to be toxic for the bacteriophage that we have studied.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CONNECTICUT VALLEY BRANCH

YALE MEDICAL SCHOOL, NEW HAVEN, Dec. 3, 1938

PRELIMINARY REPORT OF SEROLOGICAL AND CULTURAL STUDIES ON CLOSTRIDIUM HISTOLYTICUM. *George Valley and Catherine S. Flynn,* Yale University and Vassar College.

Results obtained with forty-two strains of *Clostridium histolyticum* show that surface growth on an agar medium may appear either as smooth, raised, opaque, or as irregularly branched, almost transparent colonies with slightly raised (conical) centers. A thin film may also develop. Subsurface growth in deep semisolid cysteine agar shake tubes shows small lenticular colonies in early stages; later additional units develop to form irregularly shaped coral-like colonies. Spherical to oval colonies with roughened surfaces are also observed, surrounded by a halo of very small satellite colonies. The woolly or "cotton-ball" colony frequently develops with some strains. Many of the strains yield consistently only one type of colony. It is possible that motility may play the important rôle in this phenomenon. In 1.5% agar practically all of the strains show the smooth-surfaced, irregular, coral-like colony.

The strains seem to be uniformly proteolytic and non-saccharolytic. Three or four cultures of the total of forty-two strains ferment a limited number of carbohydrates, producing both acid and gas.

Twenty antisera were prepared by injection of formolized antigens into rabbits. Agglutination tests were run with each antiserum using both formolized ("H") and phenol-alcoholized ("O") antigens. With most of the strains both "H" and "O" antigens were agglutinated to high titer (1:2560–1:5120) by the majority of the antisera; with 13.6% of the strains only the formolized antigens were agglutinated; with 4.5% of the strains neither the "H" nor the "O" antigen was agglutinated; with an additional 4.5% of strains there was agglutination of the two antigens only with an occasional serum; two of the twenty antisera failed to agglutinate the majority of the antigens, either "H" or "O".

A STUDY OF RAT PNEUMONIA. *David Borden and Walter L. Kulp,* Laboratory of Bacteriology, Connecticut State College, Storrs, Connecticut.

A small Gram-negative motile bacillus has been isolated from the lungs and heart blood of rats in the acute stage of pneumonia. This organism appears to be identical with *Bacillus bronchisepticus* both morphologically and biochemically. Serologically it has been determined that antigenic differences exist between the organism isolated from infected rats and known strains of *Bacillus bronchisepticus.* The organism in question also shares

certain antigenic components with an organism recently isolated by Eldering and Kendrick, and temporarily named by them *Bacillus para-pertussis*. Success has been obtained in 100 percent of the attempts to induce rat pneumonia by the intratracheal method of inoculation. The histopathology of the lungs of artificially infected rats appears to be identical with those of spontaneous cases. It has been found that the rat pneumonia organism, *Bacillus bronchisepticus* and *Bacillus para-pertussis* each produce a substance which is capable of oxidizing di-methyl paraphenylene diamine hydrochloride.

METHODS FOR THE DIAGNOSIS AND CONTROL OF BOVINE MASTITIS. *L. W. Slanetz and J. Naghski*, New Hampshire Agricultural Experiment Station, Durham, N. H.

Study of different tests for the diagnosis of bovine mastitis showed that there is considerable variation in the efficiency of these tests, and that their limitations must be recognized when interpreting the reactions. The strip cup, brom-thymol-blue and Hotis tests can not be relied upon for an accurate diagnosis of mastitis infection. A combination of the microscopic test, Edwards' medium and blood agar plate was very satisfactory for the detection of infected animals. It was found that the presence of leucocytes can be determined from smears of incubated samples used for the microscopic detection of streptococci. It was also demonstrated that composite samples may be employed for the diagnosis of bovine mastitis. This greatly reduces the labor, time, and materials necessary for the collection and examination of the milk samples. A plan was presented for the diagnosis and segregation of animals in a herd.

SOME FACTORS AFFECTING THE AGGLUTINABILITY OF STREPTOCOCCI ASSOCIATED WITH BOVINE MASTITIS. *W. N. Plastridge, L. F. Williams and Laura Banfield*, Department of Animal Diseases, Storrs Agricultural Experiment Station, Storrs, Connecticut.

All of the ingredients, especially the kind of peptone used in preparing nutrient broth were found to have a pronounced influence on agglutinability of udder streptococci. The percentage of usable antigens obtained from cultures grown in broths containing 0.2 percent glucose and prepared with Witte's peptone, Difco Bacto-Peptone, Armour's peptone, Difco Bacto-Tryptone, Difco Neopeptone, Fairchild's peptone and Difco Proteose-Peptone varied from 7.1 to 88.2 percent. Difco Proteose-Peptone yielded the highest percentage of usable antigens. Fresh beef infusion was decidedly superior to Savita yeast extract and commercial beef extract (Lemco). Use of phosphate buffer aided in the production of usable antigens. The optimum concentration of glucose was 0.5 percent, of sodium chloride 0.5 percent and of Difco Proteose-Peptone 8.0 percent. The optimum initial hydrogen ion concentration of buffered beef infusion broth prepared with Difco Proteose-Peptone and containing 0.5 percent glucose was pH 7.5. The optimum period of incubation was 48 hours.

Mechanical shaking and extraction with 0.5 percent "Antiformin" partly improved spontaneously agglutinable antigens. Distinctly inagglutinable or spontaneously agglutinable cells were seldom made specifically agglutinable by similar treatment.

WASHINGTON BRANCH

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., DECEMBER 20, 1938

THE SAPROPHYTIC ACID-FAST BACTERIA. *Ruth Gordon*, Bureau of Plant Industry.

METHODS FOR THE INVESTIGATION OF FOOD POISONING OUTBREAKS OF BACTERIAL ORIGIN. Symposium conducted by *O. B. Williams*, National Canners Association, assisted by *A. C. Hunter and G. L. Slocum*, Food and Drug Administration; *L. H. James*, University of Maryland; *S. E. Stuart and B. L. Levine*, National Institute of Health.

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., JANUARY 17, 1939

PSEUDOMONAS AERUGINOSA IN BOVINE MASTITIS. *J. F. Cone*, Division of Market-Milk Investigations, Bureau of Dairy Industry, U. S. Department of Agriculture.

Pseudomonas aeruginosa was apparently the sole etiological agent in two outbreaks of extremely severe mastitis. No mixed infections were found and most of the cows involved had had no previous history of mastitis. The acute febrile stage usually lasted only a few days, but with some of the cows the organisms were shed into the milk long after the secretion appeared normal macroscopically.

Eighteen strains of the organisms from mastitis and four strains of *P. aeruginosa* obtained from other laboratories were studied in pure cultures and were found to differ from Bergey's description of the species in their inability to produce indole, and in their ability to produce acid from some of the simpler carbohydrates.

TECHNIQUE AND RESULTS OF THE TREATMENT OF BACTERIA WITH MONOCHROMATIC ULTRAVIOLET RADIATION. *A. Hollaender*, National Institute of Health.

.

THE LACTASE ACTIVITY OF ESCHERICHIA COLI-MUTABILE[1]

CHARLES J. DEERE, ANNA DEAN DULANEY AND I. D. MICHELSON

Department of Chemistry and Department of Bacteriology, University of Tennessee School of Biological Sciences, Memphis

In a previous publication (Deere, Dulaney and Michelson, 1936) we reported that the non-lactose-fermenting (white) form of *Escherichia coli-mutabile* when grown in lactose broth utilizes very small quantities of lactose, if any, before the appearance of lactose-fermenting variants (reds). Appreciable lactose utilization and production of acid and gas were so closely correlated with the appearance of lactose-fermenting variants as to warrant the conclusion that the fermentative changes were due to these variants.

This earlier study has been extended to include determinations of the lactase content of cell preparations of both the white and red forms of *Escherichia coli-mutabile*. Since both forms are able to ferment monoses the difference in their cultural behavior in lactose broth must be due to a difference either in lactase content or activity.

The behavior of *Escherichia coli-mutabile* in lactose broth suggests that its lactase is an adaptive enzyme (Virtanen, 1934), viz., that lactase is produced only in the presence of lactose. Hershey and Bronfenbrenner (1936) found the non-lactose-fermenting form of a lactose-variable coli-aerogenes intermediate to be *deficient* in lactase, while its lactose-fermenting variant contained lactase. We were surprised, therefore, to find that

[1] Part of the data in this paper are taken from a dissertation presented by Charles J. Deere to the Committee on Graduate Study in partial fulfilment of the requirements for the degree of Doctor of Philosophy, University of Tennessee, September, 1937.

lactase in *Escherichia coli-mutabile* is not an adaptive enzyme, in terms of Virtanen's definition, since its presence is not dependent on growth of the organism in the presence of lactose (tables 2 and 3). Furthermore, the lactase activity of this organism in cultures is not determined directly by its lactase content.

The isolation and cultural reactions of the organism used in this study have been described (Dulaney and Michelson, 1935 and Michelson and Dulaney, 1936). The Garrett strain used in former work was employed in these studies. The white is the non-lactose-fermenting *Escherichia coli-mutabile* and the red is its lactose-fermenting variant.

METHODS

Analytical

The lactase content of cell preparations was determined by their ability to hydrolyze lactose, the change in reducing power of the substrate being measured by the Shaffer-Hartmann method as modified by Shaffer and Somogyi (1933). With Reagent No. 50 and a 15 minute period of heating, the per cent increase in reducing power of a solution of lactose follows closely the per cent of hydrolysis of lactose (fig. 1).

Cell preparation

Determination of lactase content by the above method requires cell preparations whose ability to act on monoses has been destroyed. Karstrom (1931) and Hershey and Bronfenbrenner (1936) have used toluene for inhibiting enzyme action on monoses and preventing the interference of contaminants. We have found that thymol will sterilize heavy suspensions of colon organisms within one hour, while toluene requires 2 days (Hershey and Bronfenbrenner). In other respects thymol is also more satisfactory than toluene.

In order to measure accurately the test quantities of cell preparations we have weighed samples dried by vacuum distillation at 35–38°C., having demonstrated that this procedure is innocuous to lactase.

Substrate

The substrate used was 50 cc. of 0.5 per cent lactose in 1 per cent acacia and 0.1 M phosphate buffer at pH 7.0–7.2. The presence of acacia prevents lactase inactivation by unknown factors which otherwise prevent even roughly agreeing duplicates, particularly when the quantities of cell preparations are varied.

RELATION BETWEEN REDUCING POWER AND
DEGREE OF HYDROLYSIS OF LACTOSE
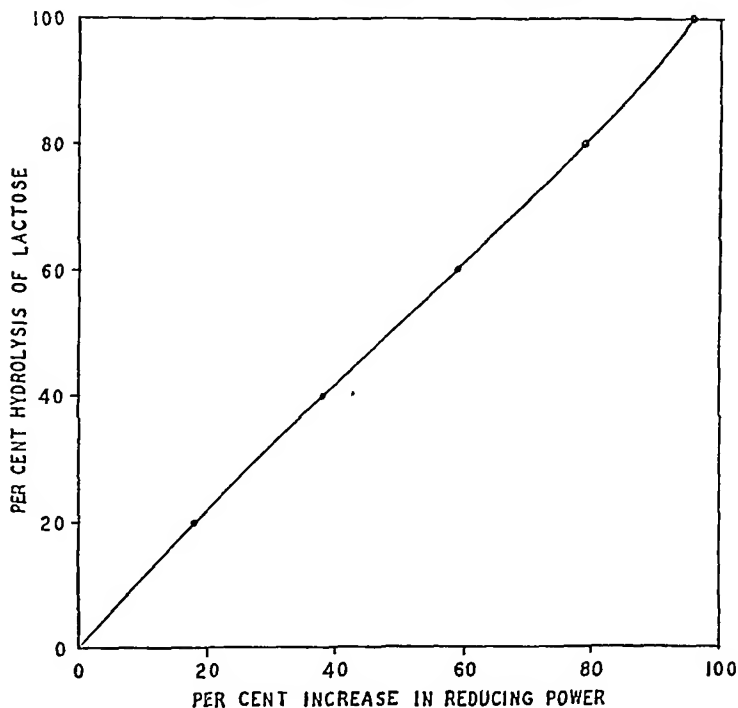
FIG. 1

The pH of the substrate is optimum for the lactase of this organism (fig. 2). For sugar determination no preliminary treatment of the system is required; the digests are simply diluted to bring the sugar content within the range determinable by the reagent.

pH determination

We have used a quinhydrone electrode for pH determinations.

Time of appearance of variants

The time of appearance of variants was determined by streaking the cultures on Endo's agar.

PH OPTIMUM FOR LACTASE
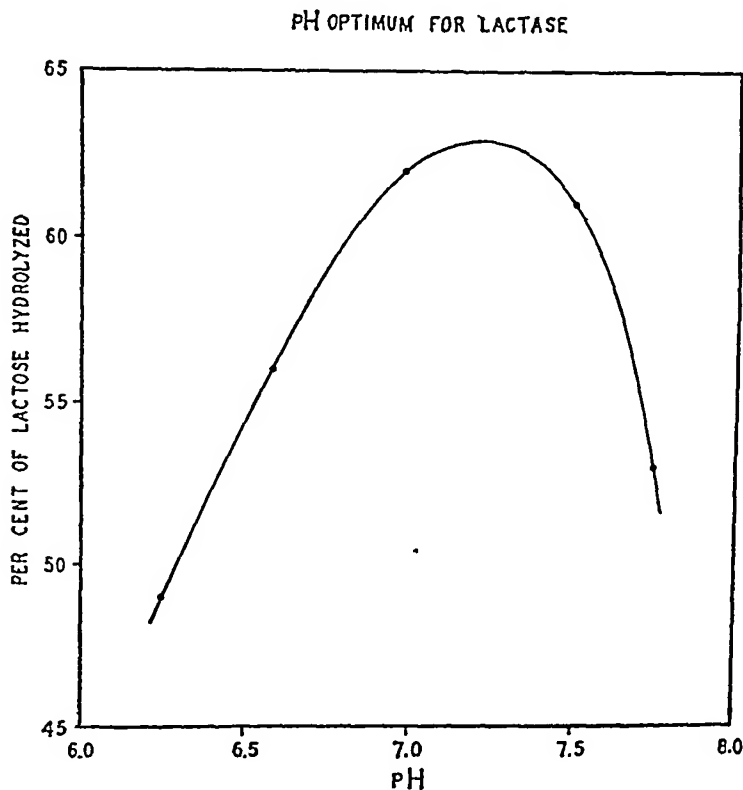
Fig. 2

EXPERIMENTAL PROCEDURE

The organisms were grown on plain or sugar agar contained in flatsided pint flasks. Ten-per-cent sugar solutions, separately sterilized, were used in the sugar agar. The growth was harvested in 8–12 cc. of saline, the cells packed by centrifugation, and the supernatant fluid used for pH determination. The cells were washed twice in distilled water and samples estimated to contain 20–30 mgm. of cells placed in weighed 100 cc. Erlenmeyer

flasks. The samples were dried rapidly by vacuum distillation with the apparatus described by Morrison and Hisey (1937) and the weight of dried material determined. The dried cells were suspended in 25 cc. of 2 per cent acacia in 0.2 M phosphate buffer, 10–20 mgm. of thymol added, and the samples placed in an incubator at 37–38°C. After periods ranging from one to one and one-half hours, 25 cc. of 1 per cent lactose were added and samples immediately withdrawn for analysis. These samples and those subsequently taken were diluted with 0.01 per cent copper sulfate in order to stop enzyme action. The reducing power of these diluted samples was determined and thereby the lactase content of the preparation estimated.

<h2 align="center">RESULTS</h2>

In our earlier work (Deere, Dulaney and Michelson, 1936) on lactose utilization of *Escherichia coli-mutabile* we employed broth cultures. Since it was desirable to use agar cultures for the present enzyme studies, a comparison of the lactase content of cells grown on the different media was indicated. The results (table 1) disclose a relatively delayed increase of lactase in cells grown on agar, and a more rapid decrease in lactase of cells in broth cultures following development of lactose-fermenting variants. These differences in behavior, however, are slight enough to justify use of agar cultures for our present purposes.

Our observations were extended to include a determination of the lactase content of cell preparations of both the white and red strains when grown on plain, glucose and lactose agar. The results are presented in tables 2 and 3. It will be observed that growth on lactose medium is not essential for the presence of lactase in either of the strains. Incidentally, the white strain used in this experiment had not been in contact with lactose for 55 days. When grown on lactose-free media the two strains have similar small amounts of lactase. Growth of either strain on lactose medium yields preparations which are many times as active as preparations obtained from lactose-free media. The white strain grown on lactose agar has about 5 times the lactase content of the red strain which has been grown on lactose-free

agar. The lactase content of the red strain when grown on lactose rises to approximately 3 times that of the white under the same conditions. The fact that a growing culture of the white strain will not ferment lactose cannot be attributed to absence of lactase.

In view of these results it seemed unlikely that increase in lactase could be the entire explanation for the development of red variants. Probably a combination of factors, rather than a single one, is responsible. The quantitative increase in lactase which occurs cannot be ignored. It may be that there is a

TABLE 1

Comparison of lactase activity of cells grown in broth and on agar

Substrate—0.5 per cent lactose, 1 per cent acacia, 0.1 M phosphate, pH 7.1.
Period of incubation in determination of lactase activity, 12 hours

	MEDIUM															Plain broth	
	Lactose agar						Plain agar				Lactose broth						
Period of growth, hours	12	24	48	72	120	240	12	24	48	72	12	24	48	72	120	24	48
pH of medium	7.2	7.3	6.5*	6.3	7.4	7.9	7.5	7.6	7.9	7.9	7.4	7.6	5.1*	4.9		7.6	7.9
Weight of sample, mgm	24.8	22.2	18.8	19.2	17.0	23.5	27.0	25.3	25.5	25.1	25.4	18.0	20.6	20.0	18.3	15.5	22.4
Per cent of lactose hydrolyzed	18	15	48	87	63	47	3	3	1	6	23	14	91	72	1	2	0
Lactase activity†	0.7	0.7	2.5	4.5	3.7	2.0	0.1	0.1	0	0.2	0.9	0.8	4.4	3.6	0	0.1	0

* Lactose-fermenting variants appeared at this interval.

† Per cent of lactose hydrolyzed per milligram of dry preparation.

critical lactase content which must be achieved before sugar utilization and acid formation sufficient to change the dye-indicator system on Endo's agar results. This critical value is acquired by the red strain during the very early hours of the growth phase. On the other hand, only certain cells of the white strain are able to ferment lactose, even after prolonged (48 hour) contact with the sugar.

Very small amounts of lactose are sufficient to cause the development of a few reds. Since the lactase content of these organisms is so greatly influenced by the presence of lactose in the medium it seemed likely that small concentrations of lacto-e

could cause the production of reds without increasing the lactase activity of the cells. This possibility was examined in the

TABLE 2

Effect of medium on lactase activity of Escherichia coli-mutabile (Garrett white)

Substrate—0.5 per cent lactose, 1 per cent acacia, 0.1 M phosphate, pH 7.1.
Period of incubation in determination of lactase activity, 12 hours

	MEDIUM											
	Glucose agar				Lactose agar				Plain agar			
Period of growth, hours........	12		24		12		24		12		24	
pH of medium.................	4.7		4.6		7.2		7.2		7.6		7.7	
Treatment of cells.............	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried
Weight of dry sample, mgm....	30.6		19.8		34.7		28.7		29.9		23.0	
Per cent of lactose hydrolyzed..............	4	6	2	4	36	37	30	30	7	4	3	4
Lactase activity*.......	0.1	0.2	0.1	0.2	1.0	1.1	1.0	1.0	0.2	0.1	0.1	0.2

* Per cent of lactose hydrolyzed per milligram of preparation.

TABLE 3

Effect of medium on lactase activity of Escherichia coli-mutabile (Garrett red)

Substrate—0.5 per cent lactose, 1 per cent acacia, 0.1 M phosphate, pH 7.1.
Period of incubation in determination of lactase activity, 12 hours

	MEDIUM											
	Glucose agar				Lactose agar				Plain agar			
Period of growth, hours........	12		24		12		24		12		24	
pH of medium.................	4.7		4.7		5.1		5.2		7.5		7.7	
Treatment of cells	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried
Weight of dry sample, mgm....	32.4		23.5		32.1		24.7		38.0		23.6	
Per cent of lactose hydrolyzed..............	0	4	5	3	90	86	70	68	4	6	7	4
Lactase activity*.......	0	0.1	0.2	0.1	2.8	2.7	2.8	2.8	0.1	0.2	0.3	0.2

* Per cent of lactose hydrolyzed per milligram of preparation.

following experiments (table 4). Garrett white was grown on agar containing 0.001, 0.01, 0.1 and 1 per cent lactose and the lactase content of the cells followed. Lactose in concentration of

0.01 per cent was sufficient to cause the production of red variants but this concentration gave a negligible increase in lactase content over that observed in organisms grown on 0.001 per cent of lactose-free agar, on which reds failed to appear. These results may indicate that increase in lactase content of the white strain is not as closely correlated with variation from white to red as we first believed (Dulaney and Deere, 1937). On the other hand, it may be argued that the particular cells of the white strain which gave rise to the reds did so by increasing their lactase content. It is well known that a great variation in rate of dissociation may be demonstrated among colonies which represent

TABLE 4

Effect of lactose concentration on lactase activity of Escherichia coli-mutabile

Substrate—0.5 per cent lactose, 1 per cent acacia, 0.1 M phosphate, pH 7.1. Period of incubation in determination of lactase activity, 12 hours

	LACTOSE CONCENTRATION IN MEDIUM											
	0.001 per cent			0.01 per cent			0.1 per cent			1.0 per cent		
Period of growth, hours	24	48	72	24	48	72*	24	48*	72	24	48*	72
pH of medium	7.6		8.1	7.6	7.7	8.0	7.6	7.6	7.4	7.4	6.9	6.4
Weight of dry sample, mgm.	26.1		26.3	25.4	27.5	26.8	28.8	28.9	23.2	28.5	27.4	23.9
Per cent of lactose hydrolyzed	6		5	6	8	7	9	21	37	28	35	51
Lactase activity†	0.2		0.2	0.2	0.3	0.3	0.3	0.7	1.6	1.0	1.3	1.8

* Lactose-fermenting variants appeared at this interval.

† Per cent of lactose hydrolyzed per milligram of dry preparation.

the descendants of one white colony. Furthermore, a few reds may be demonstrated in broth containing 0.0025 per cent lactose, though this concentration of lactose does not yield sufficient acid to produce a color change with brom-thymol-blue indicator.

The white strain may be grown on lactose agar for an indefinite period of time without giving rise to reds, provided frequent (daily) transfers are made. Under these conditions the lactase content of the cells approaches but never equals that of reds grown under the same conditions.

CONCLUSIONS

1. Lactase is present in both the white and red forms of *Escherichia coli-mutabile* when these organisms have been grown

in the absence of lactose. Lactase must therefore be classed as a constitutive enzyme, though the cultural behavior of the organisms would lead one to class its lactase as an adaptive enzyme.

2. Growth of both the white and red organisms on lactose media results in preparations several times as active in lactase as similar preparations obtained from lactose-free media. Under such conditions the lactase content of the white approaches, but never equals, that of reds grown under similar conditions.

3. The white form can be kept in contact with lactose indefinitely without the production of red forms, in spite of marked increase in lactase.

4. Dissociation to reds may occur in small degree without a measurable increase in lactase content of the entire culture.

5. Preparations of white organisms grown on lactose contain more lactase than preparations of red organisms grown in the absence of lactose.

6. Quantitative increase in lactase does not altogether explain dissociation which is probably due to a combination of factors.

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A NOTE ON THE MONOPHASIC NON-SPECIFIC SALMONELLA TYPES[1]

D. W. BRUNER AND P. R. EDWARDS

Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Ky.

The discovery of phase variation by Andrewes (1922) has had a profound effect on the differentiation of *Salmonella* types. This phenomenon and the demonstration of heat-stable and heat-labile receptors by Weil and Felix (1920) form the basis for the modern classification of the paratyphoid group. Through the work of a number of investigators it has gradually been accepted that the motile *Salmonella* strains are divisible into four groups. These are as follows:

A. Diphasic strains possessing the specific and non-specific phases of Andrewes (1922).

B. Diphasic cultures which display a variation confined to two specific antigens. This is the alpha-beta phase variation of Kauffmann and Mitsui (1930).

C. Monophasic cultures which exist only in the specific phase.

D. Monophasic cultures which exist only in the non-specific phase. These are usually considered as the non-specific counterparts of diphasic cultures.

It is with the fourth group, the forms generally regarded as being totally and permanently non-specific, that the present paper deals. This group is composed of five .varieties: Binns, Kunzendorf, Voldagsen, Berlin and Puerto Rico. It is proposed to demonstrate that these organisms are not totally non-specific but are actually diphasic and possess specific components.

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[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

METHODS

The simplest method of isolating a specific phase from a *Salmonella* strain is to plate the culture and examine the resulting colonies by slide agglutination with specific and non-specific serums. Such a procedure is quite satisfactory in strains which produce a relatively large percentage of specific colonies, but is most laborious and quite likely to result in failure when the culture under examination produces very few specific colonies. Scott (1926) and Bridges and Scott (1931) isolated specific phases from stubbornly non-specific cultures by cultivating them for several generations in broth containing antiserum derived from the non-specific phase. After this treatment, specific colonies appeared on plate cultures.

Wassén (1935) described a method for the separation of specific and non-specific phases which was based on the immobilizing effect of homologous immune serum. Filter paper impregnated with the appropriate serum was placed in semisolid agar and the medium inoculated at a point somewhat removed from the paper.

The migration of the homologous phase was checked at a distance from the paper, while the heterologous phase approached more closely. This method was improved and simplified by Gard (1937), who added the immune serum directly to semi-solid agar and inoculated the medium by stabbing. The growth of the homologous phase was confined to the line of stab while the heterologous phase diffused through the medium. This modification was used by the writers. The details of the method are as follows: Approximately 5 ml. of the semi-solid agar of Jordan, Caldwell and Reiter (1934) was placed in tubes having a diameter of 11 mm. After sterilization, the medium was cooled to 45°C. and sufficient sterile agglutinating serum derived from the Kunzendorf type added to give a final concentration of 1 to 1200. The serum had a titre of 1 to 5000 for the homologous strain. The concentration of serum must be gauged rather carefully since too little fails to confine the growth of the homologous phase to the line of stab, while too large an amount of serum immobilizes both phases through the action of somatic

agglutinins. The agar containing the non-specific serum was inoculated by a single stab at one side of the tube. A heavy line of growth appeared along the line of stab and if organisms existing in the specific phase were present they grew out through the medium, forming protuberances from the line of inoculation. By fishing from the protruding growth and reinoculating into the same medium the specific phase was obtained. At times it was necessary to transfer the culture in this manner several times before the non-specific phase was eliminated. It is helpful to transfer the culture to an agar slant also, each time it is transferred in semi-solid agar. Growth from the agar slant can be tested by slide agglutination with specific and non-specific serums, thus enabling the worker to judge the status of the culture at each transfer.

<div align="center">RESULTS</div>

The Binns type of Schütze (1920) was described by White (1926) as a totally non-specific variant of *Salmonella typhimurium*. It was found by Edwards (1936) to be diphasic. The specific phase of the orignal Binns strain was demonstrable only with the greatest difficulty by ordinary plating methods and by growth in non-specific antiserum broth. Therefore, it offered an opportunity to compare the efficiency of those methods with that of the Wassén technique. When the Binns strain was inoculated into the semi-solid agar containing non-specific serum, the whole tube of agar was turbid after over-night incubation. It was first thought that the concentration of serum was insufficient to confine the non-specific phase to the site of inoculation. However, when organisms were fished from the side of the tube opposite the line of stab, they were found to represent the specific phase and failed to agglutinate with non-specific antiserum. This was repeated several times and each time the result was the same. The results with the Binns strain demonstrate how much more effective is the Wassén technique than are other methods of isolating specific components.

The Kunzendorf variety, or western European type of *Salmonella cholerae-suis*, was found by White (1926) to be totally

non-specific. Gard (1937) mentions the fact that he obtained specific phases from Kunzendorf cultures by the Wassén technique. The two cultures to be reported upon here are type cultures (numbers 309 and 1713) of the National Collection of Type Cultures of Great Britain. These two strains were among those studied by White and found monophasic. When they were inoculated into the test medium, both displayed a small spherical projection from the line of stab after 48 hours incubation. On the second transfers the migrations from the site of inoculation were much more pronounced and on the third transfers the specific phase was isolated from both strains. These specific phases were agglutinated to the titre of specific *S. cholerae-suis* antiserum, were not flocculated by non-specific serums and were able to absorb completely the floccular agglutinins from specific *S. cholerae-suis* antiserum. In addition to the two cultures described, specific phases were also isolated from 6 other Kunzendorf strains.

The Voldagsen type of Dammann and Stedefeder (1910) was demonstrated by White (1926) to be the monophasic non-specific variant of *Bacillus typhi-suis* of Glässer (1909). Serologically, it is identical with the Kunzendorf type but differs in its very delicate growth on artificial mediums and by its rapid fermentation of arabinose and trehalose. The only strain of this type available was the Dammann strain of the National Collection of Type Cultures of Great Britain. Four tubes of semi-solid agar were inoculated with the culture. After 48 hours incubation one of them exhibited evidence of phase variation. After four transfers, subcultures from this tube yielded the specific phase. It was agglutinated to the titre of specific *S. cholerae-suis* antiserum and was not flocculated by non-specific serums.

The Berlin type was described by Kauffmann (1929) as a totally non-specific variant of the Thompson type of Scott (1926). Gard (1937) recorded the isolation of specific phases from Berlin cultures. Four cultures were available for study, 3078 of the National Collection of Type Cultures of Great Britain and three cultures received from Dr. Kauffmann. The specific phase of culture 3078 was isolated after three transfers in semi-solid agar. The remaining three cultures behaved as

did the Binns strain, the specific phase clouding the whole tube on the first transfer in the semi-solid medium. The specific phases were flocculated to the titre of specific Thompson antiserum but were not agglutinated by non-specific serums. The specific phase of the Berlin type removed all agglutinins from specific Thompson antiserum.

The Puerto Rico type was described by Kauffmann (1934) as a totally non-specific variant of the Newport type. Only one culture, the strain originally studied by Kauffmann, was available. When it was inoculated into semi-solid agar the non-specific phase spread through the medium and no specific components could be isolated. The amount of serum in the medium was then doubled and the specific phase was isolated without difficulty. The specific phase was flocculated to the titre of specific Newport antiserum, did not agglutinate with non-specific serums and removed all agglutinins from specific Newport antiserum when used in absorption tests.

DISCUSSION

The demonstration of specific components from cultures of all the so-called monophasic non-specific varieties of *Salmonella* makes extremely doubtful the existence of a true permanently and totally non-specific strain. The presence of specific phases in the organisms under discussion renders it no longer necessary to include the Binns, Voldagsen, Puerto Rico and Berlin varieties in the classification of the genus *Salmonella*. They are identical with *Salmonella typhi-murium*, *Salmonella typhi-suis*, the Newport type and the Thompson type respectively. The Kunzendorf type is serologically identical with *Salmonella cholerae-suis* but differs in its biochemical activity.

The technique of Wassén is far superior to other methods generally used for the isolation of specific phases from stubbornly non-specific races. Kauffmann (1936) discovered that the typhoid bacillus, which was long regarded as a monophasic organism, actually displays alpha-beta phase variation. It seems quite possible that by the use of this method other supposedly monophasic specific species may be found to exhibit phase variation.

CONCLUSIONS

The Wassén technique is an excellent means of isolating phases of *Salmonella* which are suppressed under ordinary conditions of culture. By its use specific phases have been isolated from all of the so-called monophasic non-specific *Salmonella* types.

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PRODUCTIVITY OF MEDIA CONTAINING MILK FOR RECENTLY ISOLATED STRAINS OF THE COLIFORM GROUP[1]

M. T. BARTRAM[2] AND L. A. BLACK

Department of Bacteriology, University of Maryland, College Park

The productivity of various media used in water analysis for the isolation of the coliform group has been frequently reported in recent literature. Since such studies may not be applicable where milk constitutes a portion of the inoculum, the Most Probable Number was determined in these experiments in a series of liquid and solid media containing milk.

The method described by Butterfield (1933) and Hoskins (1933), for determining the comparative productivity of media for coli-aerogenes organisms, was modified, in that 10 instead of 15 tubes of each medium were used and two dilutions were planted instead of three. Sterile whole milk in 1 cc. amounts was added to each tube of liquid media, except standard lactose broth, before the organisms were added. With solid media the number of organisms in the buffer infusion was determined by plating in standard agar. From the results obtained after 24 hours' incubation, two dilutions of the original suspension were plated into each of the trial media in triplicate, such dilutions being used that the plates would show between 30 and 300 colonies per plate. Sterile whole milk was added to each plate in 1 cc. quantities before introducing the suspension.

The media employed were selected from those used by us in a

previous study (Bartram and Black, 1936) and were prepared from dehydrated products or according to the methods described by their proponents.   Both the liquid and solid media were made up in such strengths that the addition of the 1 cc. of milk would serve to reduce the concentration of the medium to the usual

TABLE 1

Productivity of media containing milk for coliform organisms; results reported as percentage of most probable numbers compared to standard lactose as 100 per cent

(Average of two recently isolated strains)

MEDIA	ESCHER-ICHIA	AERO-BACTER	INTER-MEDIATE	AVERAGE
Methylene-blue brom-cresol-purple......	99	107	34	80
Fuchsin lactose.........................	71	50	79	66
Brilliant-green bile.....................	75	81	36	64
Formate ricinoleate.....................	15	36	27	26
Eijkman................................	0	0	0	0

TABLE 2

Productivity of media containing milk for coliform organisms; results reported as percentage of colonies obtained compared to standard agar plus milk as 100 per cent

(Average of two recently isolated strains)

AGAR	ESCHER-ICHIA	AERO-BACTER	INTER-MEDIATE	AVERAGE
Neutral-red bile.........................	97	121	146	121
Violet-red bile..........................	120	92	128	113
Endo....................................	86	116	103	102
Brilliant-green lactose bile..............	75	91	120	95
Taurocholate...........................	93	123	30	82
Desoxycholate..........................	92	70	20	61
Trypaflavin.............................	4	45	5	18

value.   All tubes and plates were examined at the end of 24 hours and 48 hours.   Two strains each of *Escherichia, Aerobacter* and intermediates recently isolated from milk were used.   The dilutions were so prepared that 1 cc. quantities could be used in making each inoculation and in all instances the media were inoculated alternately.

RESULTS

The results obtained are shown in tables 1 and 2, in which the percentage productivity of each liquid medium is based on standard lactose broth as 100 per cent and each solid medium on standard agar plus milk as 100 per cent. On the basis of the results obtained, the liquid media would be placed in the following order, the most productive first: (1) methylene-blue bromcresol-purple; (2) fuchsin lactose; (3) brilliant-green bile, and (4) formate-ricinoleate. The Eikjman medium was unsatisfactory, giving negative results in all tests with the dilutions used. The solid media would rank as follows: (1) neutral-red bile; (2) violet-red bile; (3) Endo; (4) brilliant-green lactose bile; (5) lactose taurocholate; (6) desoxycholate, and (7) trypaflavine agar.

DISCUSSION

These experiments may not be sufficiently extensive to evaluate completely the various media and for such a purpose a more detailed examination may be necessary, as pointed out by Noble (1935). However, the results may be used within recognized limits, especially since no "border-line" medium was indicated. The results are somewhat in disagreement with those obtained in water analysis by other workers, none of whom employed milk with the inoculum. Ruchhoft (1935) in summarizing data received from coöperating laboratories, using the method of Butterfield-Hoskins, obtained the following productivity from highest to lowest: buffered lactose, fuchsin lactose, methylene-blue bromcresol-purple, brilliant-green bile, crystal violet and formate-ricinoleate. Ruchhoft and Norton (1935) in a preliminary report obtained essentially the same results except that the positions of buffered lactose and fuchsin lactose were reversed. Farrell (1935) rated buffered lactose and brilliant-green bile, in the order named, ahead of fuchsin lactose and placed trypaflavin broth below formate-ricinoleate. In this laboratory, Black and Klinger (1936) observed buffered lactose, fuchsin lactose, methylene-blue bromcresol-purple, brilliant-green bile, crystal-violet and formate-ricinoleate to rank in the order named. These results agree with

those found by Ruchhoft. It would be interesting to determine if the addition of 1 cc. of milk caused the change in relative productivity of fuchsin lactose and methylene-blue brom-cresol-purple.

As far as is known, the productivity of the various solid media has not been reported so that no comparison is possible. However, the results in general confirm those obtained in previous work by us, (Bartram and Black, 1936) although desoxycholate agar fell below the results that might have been expected. This was accounted for by its low productivity with the strains of the intermediate group and may be characteristic of the medium, although this has not been determined with additional intermediate strains.

SUMMARY

Two strains each of *Escherichia*, *Aerobacter*, and intermediates recently isolated from raw milk were inoculated into five liquid and seven solid media in addition to standard lactose broth and standard methods agar, employed as controls.

A modification of the method of Butterfield was followed with two dilutions inoculated into 10 tubes of each medium. Two dilutions were plated in triplicate in the solid media and 1 cc. of sterile whole milk was added to each plate or tube before adding the organisms.

The productivity of the liquid media tested, based on Hoskins' Most Probable Numbers was, in order of descending sensitivity: (1) methylene-blue brom-cresol-purple; (2) fuchsin lactose; (3) brilliant-green bile; (4) formate-ricinoleate; (5) Eijkman medium. No one of the media was as productive as standard lactose.

The solid media ranked as follows: (1) neutral-red bile; (2) violet-red bile; (3) Endo; (4) brilliant-green lactose bile; (5) lactose taurocholate; (6) desoxycholate, and (7) trypaflavine agar. The unsatisfactory results obtained with desoxycholate agar may be accounted for by its low productivity with the cultures of the intermediate group used. Neutral-red bile and violet-red bile gave counts considerably higher than those obtained on standard agar plus milk.

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CHEMICAL FACTORS INFLUENCING THE GROWTH OF TUBERCLE BACILLI[1]

I. Metal Catalysts

BEN C. SHER AND HENRY C. SWEANY

Research Laboratory of the City of Chicago Municipal Tuberculosis Sanitarium

INTRODUCTION

This investigation deals with the effects of cupric, manganous, ferric, and antimonate ions; extracts of calves' lungs, calves' hearts, and chicken livers; and of diphenylamine and other organic compounds; on the growth of H37, a human strain of tubercle bacilli. It was carried out at the suggestion of Dr. Kharasch, who, with his co-workers (Kharasch, Conway and Bloom, 1936), had studied the effects of the metal ions of copper, iron, and manganese as well as other compounds and had observed the response of the organisms in question toward these reagents.

To permit the greatest possible control of the factors involved, the bacilli were grown on Long's synthetic medium, (Long and Seibert, 1926) from which the ferric ammonium citrate was omitted. Long's medium is prepared by autoclaving a solution of the following in one liter of distilled water:

	gm.
Asparagin	5
Ammonium citrate	5
KH_2PO_4	3
Na_2CO_3	3
$MgSO_4 7H_2O$	1
NaCl	2
Glycerol	50
Ferric ammonium citrate	0.05

[1] Presented before the Division of Biological Chemistry of the American Chemical Society at the Pittsburgh meeting, September, 1936.

The solution is then adjusted to a pH of 7.2–7.4 with hydrochloric acid.

The ferric ammonium citrate, or other iron salts, when used in this investigation are considered as additional substances, in the same sense as are cupric or manganous salts.

It is important to note the composition of this medium in order to evaluate the effects of the constituents on metal catalysis. Kharasch, *et al.*, (Kharasch, Legault, Wilder and Gerard, 1936) have shown, in the case of the oxidation of a simple thiol system to dithiol, that the rate of oxidation is accelerated by the addition of manganese, copper, or iron salts and that their effectiveness is in the order listed. The acceleration due to the manganese is suppressed, and that due to copper catalysis is enhanced, by phosphate ions, to a degree depending upon the concentration and upon whether the phosphate ion is present as ortho-, meta-, or pyrophosphate. Glycerol inhibits the catalytic effect of iron, manganese, and copper ions. The carbonate ion probable does not affect these systems. If there is any effect, it is unchanged with different concentrations. It should also be noted that beef extract was found to inhibit the effect of iron and that liver extract inhibited both iron and copper catalysis, while the catalytic effect of manganese was not inhibited by either of these factors. The presence of dithiol in the system at the start hastened the oxidation of the thiol systems.

Similar effects are undoubtedly important in controlling the growth of bacteria on a solution such as Long's medium. However, the complexity of such a solution, particularly after it is autoclaved, or after bacterial growth has started, obviates any *a priori* statement as to the influence of additional substances on the growth of the bacilli. It is not known in what forms the phosphate ion is present, nor whether glycerophosphates are formed from the glycerol in the presence of these phosphates, nor what is the nature of the complexes which are formed from ammonium citrate, asparagin and glycerol. It has been determined that with traces of additional substances, there is a profound quantitative change and also a marked effect on the character of the growth of certain organisms. For example, the growth of

Chlorella vulgaris, on a synthetic medium (Meier, 1932) of inorganic salts, arginine and agar, which ordinarily does not take place in the presence of minute traces of copper, was unimpaired when liver extract was added to such a medium which contained copper (Kharasch, Conway and Bloom, 1936). The resultant abundant growth was dark orange instead of green, and, microscopically, the relatively large green alga had changed into a small spherical body. These striking changes caused Dr. Kharasch to suggest the study of bacteria under similarly modified conditions of growth.

PURIFICATION OF REAGENTS

For this investigation, the media are divided into two classes. In one, the chemicals used are "C.P." or "Reagent" grade (hereafter to be called "C.P." media) and in the other, the chemicals are purified to the extent that the traces of impurities are reduced to a practical minimum (hereafter called "Purified" media).

The water used for recrystallizing the reagents, for cleansing the glassware, and for the media was double distilled. The second distillation was carried out in an all "pyrex" glass still.

Asparagin was recrystallized four times from boiling water. The combined filtrates yielded upon evaporation a green-colored crop of asparagin which indicates the probable presence of chlorophyl-like impurities.

Potassium dihydrogen phosphate was recrystallized three times from boiling water.

Sodium carbonate was prepared by fusing purified sodium bicarbonate. The sodium bicarbonate was recrystallized three times from water between 50° and 60°C.

Ammonium citrate was purified in earlier experiments by recrystallizing the ammonium citrate twice from water. In subsequent experiments, citric acid was recrystallized and gaseous ammonia, washed through a train of three wash bottles, was passed through the solution of redissolved crystals.

Magnesium sulfate was recrystallized once from water.

Sodium chloride was prepared by passing purified hydrogen

chloride into a solution of sodium chloride. The precipitated salt was washed with water and dried.

Glycerol was distilled under reduced pressure from an all "pyrex" still.

Agar agar was used wherever solid media were required. For purified reagents, the agar was washed several times with water, and most of the water removed each time. The washed agar was then dialyzed in a cellophane bag for two days against double distilled water.

All the glassware used in preparing the media, the media flasks, and culture tubes were of "pyrex" glass. All glassware, in the earlier experiments, was cleaned with chromic-sulfuric acid cleaning solution. In later experiments, trisodium phosphate was used. In either event, after soaking 24 hours in the cleaning solution, the apparatus was washed thoroughly with hot tap water, and with distilled water and then allowed to stand in distilled water twice for 24-hour periods. The containers were plugged with cotton, then sterilized in an autoclave at 15 pounds steam pressure.

EXPERIMENTAL RESULTS

In several series of cultures, the growths were more abundant and appeared earlier on "C.P." media than on each similar modification of medium prepared with purified reagents. The "Purified" media required the addition of other substances to yield such abundant and accelerated growths. This demonstrates that the catalytic effect of the impurities present in "C.P." and reagent grade or similar quality chemicals is sufficiently great to influence and mask the effects of the traces of substances of interest in this investigation.

Table 1 was compiled from three series of cultures (H37) in each of which there were several flasks of the same modification of media. A number of interesting facts are brought out by this study. The effects of the non-ferrous catalysts on purified media, singly or in combination with each other, were not decisive. The addition of ferric ions alone did not promote growth, but the addition of ferric ions to any of the media containing the

non-ferrous catalysts singly, or in combinations, resulted in a re-markably enhanced growth. Therefore, the non-ferrous cata-lysts appear to have stimulated the action of the iron. It is

TABLE 1

The influence of metal catalysts on growth of tubercle bacilli (H37)

"PURIFIED"[*] MEDIA NUMBER	Cu	Mn	$K_4Sb_2O_7$, (H_2O)	Fe	GROWTH[‡]
	mgm. per 1000 cc.	mgm. per 1000 cc.	mgm. per 1000 cc.	mgm. per 1000 cc.	
1					1
2	0.033				1
3		0.10			1
4			30.0		2
5	0.033	0.10			1
6	0.033		30.0		2
7		0.10	30.0		2
8	0.033	0.10	30.0		2
9				7.5	1
10	0.033			7.5	3
11		0.10		7.5	3
12			30.0	7.5	5
13	0.033	0.10		7.5	4
14	0.033		30.0	7.5	5
15		0.10	30.0	7.5	5
"C.P."[†] MEDIA NUMBER					
16					3
17	0.033				4
18		0.10			4
19			30.0		4
20	0.033	0.10			4
21	0.033		30.0		5
22		0.10	30.0		5
23	0.033	0.10	30.0		4

* "Purified" media were prepared from reagents which were carefully purified.
† "C.P." media were prepared from "C.P." or reagent grade chemicals.
‡ The growths were evaluated by inspection, and classified into five groups. Five (5) represents the most abundant growth observed, and one (1) the sparsest growth.

consistent, then, that "C.P." or unpurified medium required no additional substance to yield enhanced growths, since minute traces of iron and other substances are already present in this

TABLE 2

The influence of metal catalysts on growth of tubercle bacilli (H37)

"PURIFIED" MEDIA NUMBER	Cu	Mn	K₄Sb₂O₇, (H₂O)	Fe	NUMBER OF FLASKS	AVERAGE WEIGHT OF GROWTH*	RANGE OF pH*
	mgm. per 1000 cc.	mgm. per 1000 cc.	mgm. per 1000 cc.	mgm. per 1000 cc.			
1					8	0.18	5.4 6.2
2	0.03				4	0.18	5.0 6.2
3		0.10			4	0.19	5.2 5.8
4			20.0		4	0.14	5.0 5.4
5	0.03	0.10			4	0.19	5.0 5.6
6	0.03		20.0		4	0.18	5.0 5.4
7		0.10	20.0		4	0.15	5.2 5.6
8	0.03	0.10	20.0		4	0.23	5.4 6.0
9				7.5	4	0.46	7.2 7.4
10				15.0	5	0.41	7.2 7.6
11	0.03			7.5	5	0.41	7.0 7.2
12	0.03			15.0	7	0.39	7.0 7.6
13		0.10		7.5	3	0.48	7.4
14		0.10		15.0	5	0.45	7.2
15			20.0	7.5	5	0.44	7.2 7.4
16			20.0	15.0	5	0.44	7.2 7.4
17	0.03		20.0	7.5	4	0.44	7.2 7.4
18	0.03		20.0	15.0	10	0.40	7.0 7.4
"C.P." MEDIA							
19					9	0.47	5.4 6.0

* The determinations of weights and pH were made five months after inoculation of the media. The "mother" culture was grown on Long's synthetic media for 17 days.

medium as impurities.   It is noteworthy, although not indicated in the tabulations, that, where potassium pyroantimonate is present, growths appeared earlier and were more abundant than on similar media containing no pyroantimonate.   This is also true of manganese salts although the effect is less marked.

It will be seen in table 2 that the weights of the growths obtained on "C.P." media and on "Purified" media containing iron, were more than twice the weight obtained from the analogous iron-free "Purified" media.   It is to be recalled from table 1 that "Purified" medium containing iron and no other additional substance did not consistently yield enhanced growths.   These differences may be attributed to some variations, either in technique or the introduction of minute traces of other metals.

No attempt was made in this study to investigate the pH-time relationship of the cultures, but some pH determinations which are of interest were made at the end of certain periods of growth. Five months after inoculation, "Purified" media which contained iron showed a pH which ranged from 7.0–7.6, while the pH range of the iron-free media was from 5.4–6.2.   That of "C.P." media was from 5.4–6.0 (table 2).

In another series, the results were different, in that the pH of the substrates from a $10\frac{1}{2}$ weeks' growth on "Purified" medium was 7.2 regardless of whether iron was added or not.   In this latter series, the substrates from the "C.P." media varied from 5.4–6.0.

It is generally accepted that when a different medium is to be used in culturing bacteria, in order to obtain good growths, it is frequently necessary to allow the bacteria to adapt itself to the new environment.   It is conceivable then, that the history of growth and the subsequent nature of the cultures used in transplanting may in some way be responsible for the differences observed.   Thus, the cultures indicated in table 2 were transplanted from a 17-day old growth on Long's synthetic medium, while those indicated in table 3 were made from a 15-week old culture grown on Long's synthetic medium.

In order to substantiate whether differently grown cultures of the same strain yielded different results, the following experi-

ments were carried out.   A culture of H37, which was grown on
"Purified" media for 18 weeks, was used to prepare two sub-
cultures.   For one of these, "Purified" media was used (flask a);
for the other, "C.P." media, to which iron solution was added,
(flask b).   These subcultures were allowed to grow for four weeks
and were then used for transplanting on the following media:
"Purified" alone, "Purified" containing iron, "C.P." alone, and
"C.P." medium to which iron had been added.   In each instance,
the cultures from flask b yielded earlier growths, and, inter-
estingly, the pH values of the solutions in these flasks were lower
than those from flask a (table 4).

TABLE 3

The pH of cultures when inoculated from a "mother" culture 15 weeks old

"PURIFIED" MEDIA NUMBER*	Fe ADDED	NUMBER OF FLASKS	RANGE OF pH†
	mgm. per 1000 cc.		
1	0	5	7.2
9	7.5	7	7.2
"C.P." MEDIA NUMBER			
19	0	3	5.8–6.0
19 Fe	7.5	4	5.4–6.0

* The media used were reserved from the experiments described in table 2.
† The determination of pH were made 2½ months after inoculation of the
media.   The "mother" culture was grown on Long's synthetic media for 15 weeks.

The following experiments also show that there is a difference
due to the type of inoculum used.   Two emulsions of B599 (a
nonpathogenic, rapidly growing bovine strain of tubercle bacilli)
were prepared with a mechanical emulsifier devised in our labora-
tory and described by Corper and Cohen, (1936).   One emulsion,
(Emulsion A) was prepared from a 2-week old culture grown on
"C.P." agar medium, and the other, (Emulsion B) was prepared
from a 3-day old culture which had been grown on "Purified" agar
medium through two generations.   These were used to seed
variations of "Solid Purified" medium which were prepared by
adding 2 per cent of purified agar to "Purified" medium in which
different concentrations and combinations of the following sub-

stances were incorporated: ferric ammonium citrate, manganous sulfate, vitamin C, calves' lung extract, calves' heart extract, chicken liver extract, ethylenediamine, and copper sulfate. These variations were prepared in quadruplicate and were then divided into two sets of duplicates.   The first set was inoculated with Emulsion A, the second set with Emulsion B.   *In each instance, growths appeared earlier in the first set.*

The addition of ferric ammonium citrate or other substances, did not promote growth on this agar medium, but when copper sulfate was used as the additional substance, it was found that

TABLE 4

Influence of growth history of culture on growth and pH of subcultures (H37)

"PURIFIED" MEDIA NUMBER	Fe	NUMBER OF FLASKS SEEDED FROM FLASK a	NUMBER OF FLASKS SEEDED FROM FLASK b	GROWTH AT 26TH DAY	pH
	mgm. per 1000 cc.				
1a		4		2	7.1
1b			3	5	6.9
2a	7.5	4		3	7.3
2b	7.5		3	4	6.0
"C.P." MEDIA					
3a		3		4	7.2
3b			4	6	6.8
4a	7.5	3		5	6.9
4b	7.5		4	6	6.0

The culture of H37 in flask *a* was grown on "Purified" media, that in flask *b* was grown on "C.P." media, to which iron was added.   Each was inoculated from the same culture, which was grown on "Purified" media for 18 weeks.

in the first set, the initial growth was retarded and ultimate growth impaired at concentrations of 1:24,000 to 1:48,000, while there was no growth at all with a concentration of 1:6,000. In the second set, copper sulfate inhibited only the initial growth, while ultimate abundant growths were obtained with a concentration of 1:3,000, the highest concentration used.

These data prove that the history of the growth of the "mother" cultures of tubercle bacilli is most important.

In these experiments, we also used diphenylamine as an additional substance, and have observed that a concentration of

1:20,000 prevented growth of tubercle bacilli. A concentration of 1:135,000 retarded initial growth and yielded ultimate sparse growths. This is of particular interest because similar effects were obtained by Kharasch, *et al.* (Kharasch, Conway and Bloom, 1936) in the case of *Staphylococcus aureus, Pseudomonas pyocyanea, Serratia marcescens, Torula rosea, Sarcina lutea, Spirillum rubrum*, and *Aspergillus niger*, on the different media they used.

No attempt was made to study the morphology of the bacilli, their pathogenicity, or immunological effect in the present work, and no study was made of the products elaborated. The primary object was to make a preliminary survey to determine where consistently decisive and controllable trends could be obtained. It was our experience that many factors, only some of them controllable, had an effect on the growth of tubercle bacilli. The manner in which diphenylamine exerted its effect, however, is positive irrespective of the extraneous and inherent factors involved. Its inhibitory action was decisive (although quantitatively different) with all of the media used in this investigation. This is of interest; and further results obtained with diphenylamine and similar compounds will appear in a subsequent paper.

SUMMARY

1. Long's synthetic medium which was prepared from "C.P." or "Reagent" grade chemicals, and to which no iron was added, yielded more than twice as much growth of H37 as "Purified" Long's medium, which was prepared from purified reagents.

2. The addition of iron salts to "C.P." medium, i.e., Long's synthetic medium from which iron was omitted, promoted growth only slightly.

3. Non-ferrous catalysts added to "Purified" medium did not promote growth. The addition of iron salts to "Purified" medium, particularly in conjunction with copper, manganese, or pyroantimonate ions, either singly or in combinations, yielded more than a twofold enhancement of growth of H37, as compared with "Purified" media alone.

4. Copper sulfate in minute traces retarded initial growth, and in concentrations greater than 1:3,000, prevented growth altogether.

5. Potassium pyroantimonate yielded earlier and more abundant growths.

6. Manganous sulfate yielded similar results.

7. The pH of the substrates of the cultures and the early appearance of growth appear to be controlled by the growth history of the inoculating·culture as well as the nature of the media used.

8. Diphenylamine retarded initial growth in high concentrations around 1:150,000, and prevented ultimate growth in concentrations greater than 1:20,000 in all media used.

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IMMUNOLOGICAL RELATIONSHIPS OF POLYSAC-
CHARIDES OF MUCOID ORGANISMS OF THE
TYPHOID-SALMONELLA GROUP

H. R. MORGAN AND T. D. BECKWITH

Department of Bacteriology, University of California at Los Angeles

The work of Furth and Landsteiner (1928), (1929); White
(1929), and Boivin, Mesrobeanu and Mesrobeanu (1933a), has
indicated that polysaccharide extracts obtained from *Salmonella*
will yield precipitations with homologous antisera. These tests
show cross reactions paralleling those of agglutination and con-
forming to the antigenic classification of *Salmonella* based on the
somatic, O antigens (White, 1926; Topley and Wilson, 1936;
Kauffmann, 1937). Our interest in examining the antigenic
relationship of polysaccharide materials extracted from various
Salmonella developed from finding a method for obtaining readily
the mucoid phase (Beckwith and Morgan, 1937a). This tech-
nique was found to be applicable to strains of *Eberthella typhosa*
for production of heavily encapsulated organisms (Beckwith
and Morgan, 1937b) and thus a higher content of polysaccharide
became available.

This study has included an attempt to determine the antigenic
relationships of polysaccharides extracted from two strains of
E. typhosa and one strain each of *Salmonella paratyphi A, S.
paratyphi B, S. paratyphi C, S. aertrycke, S. enteritidis, S. pullorum*
and *S. suipestifer.*

MATERIALS AND METHODS

The nine cultures used were smooth stock strains grown on
nutrient agar slants and stored in the refrigerator. Before using,
biochemical reactions were checked and compared with the re-
actions as given in Topley and Wilson (1936).

To obtain growth of mucoid character, agar slant cultures were transferred daily three times with incubation at 37°. They, then, were streaked to 1 per cent glucose beef-agar plates, and these were incubated at 16°C. for four days. The mucoid growth was suspended in physiological saline containing 1:10,000 merthiolate.

Antisera for the mucoid antigens were prepared by injecting adult, albino rabbits intravenously at 2-day intervals for a period of six weeks until animals had received a total of 14 cc. of an antigen containing 2×10^9 organisms per cubic centimeter. Blood was obtained one week after the last immunizing dose and the serum prepared.

AGGLUTINATION TESTS

For agglutination, an antigen of organisms in the smooth phase was used, as mucoid organisms are stated by White (1926) to be clumped only with difficulty by the homologous antisera. Cultures of the nine organisms were grown on nutrient agar in Blake bottles for 48 hours at 37°C. and then suspended in physiological saline containing 1:10,000 merthiolate. The suspensions were diluted to uniform turbidity.

One-half cubic centimeter of each of the nine antigens was added to $\frac{1}{2}$ cc. of dilutions of serum which ranged from 1:10 to 1:1280. Readings for complete and partial agglutination were made after two hours in a 37°C. water bath and then overnight in the refrigerator. The observations of these tests are summarized in table 1. The two animals injected with the antigen of *Salmonella pullorum* showed no production of antibodies.

POLYSACCHARIDE EXTRACTION

The method for obtaining the polysaccharide is a modification of the technique of Heidelberger, Kendall and Scherp (1936) who worked with pneumococci. Instead of using 1.2 volumes of 95 per cent alcohol to precipitate the polysaccharide, 4 volumes were employed, as the work of White (1929) indicated that this concentration was necessary in order to precipitate the specific substance of *Salmonella aertrycke, S. enteritidis, S. suipestifer* and *E. typhosa.*

Mucoid growth of the nine organisms was suspended in physiological saline containing 1:10,000 merthiolate and poured into a flask containing glass beads. The suspension remained in an incubator at 56° for 3–6 hours and then was shaken for 1 hour mechanically. The emulsion was centrifugalized at high speed for ½ hour and the precipitate of cells discarded. The supernatant was poured into a large flask. To 50 cc. of this supernatant, 0.5 gram of sodium acetate was added and then 4 volumes of 95 per cent alcohol, slowly and with continuous shak-

TABLE 1

Cross agglutination reactions

ANTIGENS	DILUTION OF ANTISERA								
	E. typhosa (T1)	*E. typhosa* (T8)	*S. para A*	*S. para B*	*S. para C*	*S. aertrycke*	*S. enteritidis*	*S. suipestifer*	*S. pullorum*
E. typhosa (T1)..........	1,280	1,280	160*	10	10	10	80	0	0
E. typhosa (T8)..........	640	1,280	20	10	10	10	80	0	0
S. paratyphi A..........	40*	20*	1,280	80	0	10	10	0	0
S. paratyphi B..........	0	0	20	640	10*	80	0	0	0
S. paratyphi C..........	0	10*	0	0	80	10*	0	80	0
S. aertrycke.............	0	0	40	160	0	1,280	0	0	0
S. enteritidis.............	40*	80*	20	0	0	0	160	0	0
S. suipestifer............	0	0	0	0	20	0	0	80	0
S. pullorum..............	80	40	20	0	0	0	80	0	0

Figures represent the highest dilution of serum that gave complete agglutination after standing overnight in the refrigerator.

0 indicates no agglutination obtained.

* Partial agglutination only obtained to this dilution.

ing. The mixture was placed in the refrigerator overnight, after which most of the alcohol was decanted. The suspension was centrifugalized, the supernatant discarded and the precipitate of polysaccharide resuspended in physiological saline. To 50 cc. gray and opalescent suspension of the polysaccharide 2 grams of sodium acetate were added and the fluid was made faintly acid to litmus by glacial acetic. Four volumes of 95 per cent alcohol were added to reprecipitate the polysaccharide. This resuspension and reprecipitation were repeated three times

for purification. The polysaccharide was finally suspended in physiological saline containing 1:10,000 merthiolate.

These colloidal solutions of the polysaccharide complexes gave faintly positive biuret and strongly positive ninhydrin tests which indicate the presence of peptide linkages. Millon and Hopkins-Cole tests were negative. Esbach (picric acid) and Exton (sulpho-salicylic acid) tests for intact protein were negative. The Molisch test for carbohydrate was strongly positive. The iodine test for glycogen was negative. These tests were

TABLE 2

Cross precipitation reactions of polysaccharides

ANTIGENS DILUTED 1 500	ANTISERA DILUTED 1 2.5								
	E. typhosa (T1)	*E. typhosa* (T8)	*S. para A*	*S. para B*	*S. para C*	*S. aertrycke*	*S. enteritidis*	*S. suipestifer*	*S. pullorum*
E. typhosa (T1)	+++	++++	+	+	±	−	±	−	−
E. typhosa (T8)	+++	++++	++	+	±	±	++	−	−
S. paratyphi A	+	+	+++	+	−	±	±	−	−
S. paratyphi B	+	+	++	++	−	+++	−	−	−
S. paratyphi C	±	±	−	−	++	−	−	++	−
S. aertrycke	−	−	+	+	−	+++	−	−	−
S. enteritidis	++	+	+	±	−	−	+++	−	−
S. suipestifer .	−	−	−	−	++	−	−	+++	−
S. pullorum .	+++	+++	+	+	−	−	++	−	−

− to ++++ indicate the degree of precipitation; ± precipitation recorded with aid of hand lens.

carried out on solutions of polysaccharide complexes containing from 4.3 to 13.5 milligrams per cubic centimeter. The intensity of the biuret test varied directly with the concentration of polysaccharide present in the solution.

The amount of polysaccharide in each of the solutions was determined by the weight of the residue when a measured volume was dried for 24 hours at 80–90°C. Dilutions of 1:500, 1:1000 and 1:5000 by weight were prepared. The immune sera were diluted to 1:2.5 with physiological saline. To $\frac{1}{2}$ cc. of the diluted serum, $\frac{1}{2}$ cc. of the various dilutions of antigens were added.

As controls, $\frac{1}{2}$ cc. of the 1:500 antigen was added to $\frac{1}{2}$ cc. of normal rabbit serum and to $\frac{1}{2}$ cc. of saline. The tubes were shaken and placed in a water bath at 37°. Readings for the amount of precipitate formed were made at the end of 2 hours at 37° and after storage overnight in the refrigerator. The amount of precipitation was recorded as $\pm$ to $+++$. To summarize the results, table 2 records the amount of precipitate in the tube containing the 1:500 dilution of antigen.

The results of the cross agglutination and cross precipitation tests seem to justify the conclusion that the protein-free polysaccharides obtained by the extraction of mucoid cultures of *E. typhosa, S. paratyphi A, B* and *C, S. aertrycke, S. enteritidis, S. suipestifer* and *S. pullorum* represent a portion at least of the surface antigen of the organisms. The polysaccharide complex seems to contain all of the major factors of the O antigen as the cross reactions obtained in the agglutination tests are confirmed by the precipitation reactions. Both of these series conform to the cross reactions expected on the basis of the White-Kauffmann antigenic classification of *Salmonella* based on the O surface antigens.

EXPERIMENTS USING ANTISERA PREPARED AGAINST POLYSACCHARIDE EXTRACTS

Using various methods for extraction of the polysaccharide complex, Boivin, Mesrobeanu and Mesrobeanu (1933b); Raistrick and Topley (1934), Morgan (1937) and Henderson and Morgan (1938), prepared extracts from *S. aertrycke, Shigella dysenteriae* (Shiga) and *E. typhosa* which would produce agglutinins for the cells and precipitins for the polysaccharide complexes when used to immunize animals. These extracts yielded negative outcomes for intact protein but usually gave positive biuret and ninhydrin tests and a strongly positive Molisch reaction. The chemical analyses of Boivin, Mesrobeanu and Mesrobeanu (1933b) and Morgan (1937) indicate that these substances are polysaccharide-lipoid complexes; the polysaccharide is the soluble specific substance which had been isolated in earlier work.

Our material seems to have some of the same characteristics as the antigenic extracts of the above investigators. To determine whether our preparations were antigenic, adult albino rabbits were immunized with each of the nine extracts. Each animal received the same weight which was as follows: intracutaneously, 13.7 mgm.; subcutaneously, 17.5 mgm.; intraperitoneally, 35 mgm. and intravenously, 10 mgm. This totals 76.2 mgm. The earlier injections were administered intracu-

TABLE 3

Cross agglutination reactions using antisera prepared with polysaccharide extracts

ANTIGENS	DILUTION OF ANTISERA								
	E. typhosa (T1)	*E. typhosa* (T8)	*S. para A*	*S. para B*	*S. para C*	*S. aertrycke*	*S. enteritidis*	*S. suipestifer*	*S. pullorum*
E. typhosa (T1)..............	640	640	20*	10*	10*	10*	40	0	40
E. typhosa (T8)..............	320	640	40*	10*	40*	10*	20	0	20
S. paratyphi A..............	40*	40*	80	40*	0	0	10	0	10
S. paratyphi B........	20*	20*	80*	80	0	40	20*	0	10*
S. paratyphi C..............	0	0	0	0	160	0	0	80	0
S. aertrycke................	0	0	0	10	0	80	0	0	0
S. enteritidis................	40*	10	0	0	0	0	160	0	20
S. suipestifer................	0	0	0	0	40	0	0	80	0
S. pullorum.................	160	160	10*	10*	0	10*	80	0	80

Figures in the table represent the highest dilution of serum that gave complete agglutination after standing overnight in the refrigerator.

* Partial agglutination only obtained to this dilution.

0 indicates no agglutination obtained.

taneously and subcutaneously because the material was toxic to the animals.[1]

A week following the final immunizing dose, the animals were bled and serum prepared. All treated animals showed production of agglutinins and precipitins.

AGGLUTINATION TESTS

Agglutination tests were made using the technique outlined previously. Table 3 summarizes the results.

[1] Two adult albino rabbits receiving 2 mgm. of extract of *E. typhosa* intravenously died within 3 hours.

Cross precipitation tests were set up as before using the antisera prepared with the polysaccharide extracts. The precipitations are summarized in table 4.

The precipitation tests give striking confirmation of the cross reactions obtained by agglutination. The antisera prepared with the antigenic polysaccharide extracts give the same cross reactions as those obtained with antibacterial sera. The extract therefore must contain the O antigens of the cells.

TABLE 4

Cross precipitation reactions using antisera prepared with polysaccharide extracts

ANTIGENS DILUTED 1:500	ANTISERA DILUTED 1:2.5								
	E. typhosa (T1)	*E. typhosa* (T8)	*S. para A*	*S. para B*	*S. para C*	*S. aertrycke*	*S. enteritidis*	*S. suipestifer*	*S. pullorum*
E. typhosa (T1)...	++++	+++	±	+	±	±	++	−	+++
E. typhosa (T8)...	+++	++++	±	+	−	+	++	−	+++
S. paratyphi A...	+	+	++	+	−	+	−	−	+
S. paratyphi B....	+	±	+	++	+	++	−	−	−
S. paratyphi C....	±	−	−	−	+++	−	−	++	−
S. aertrycke......	−	−	−	++	−	+++	−	−	−
S. enteritidis.....	++	+++	−	±	−	−	+++	−	+
S. suipestifer.....	−	−	−	−	+++	±	−	+++	−
S. pullorum......	++	+++	±	±	−	+	++	±	++++

− to ++++ indicate degree of precipitation after standing overnight in refrigerator; ± precipitation recorded with aid of hand lens.

SKIN REACTIONS

Animals immunized with bacterial antigens have been found to show skin sensitivity to homologous polysaccharides. Zinsser and Parker (1923) noted that animals immunized with intracutaneous injections of *E. typhosa* presented cutaneous reactions after the injection of an extract of *E. typhosa*. This material was precipitated by alcohol and gave negative biuret, Millon Hopkins-Cole and sulpho-salicylic acid tests for protein. Francis and Tillet (1931) found that animals immunized against pneu-

mococci demonstrated type-specific cutaneous reactions when the specific capsular polysaccharides were injected.

Each of the animals immunized with bacterial antigens received intracutaneously 0.3 cc. of a preparation containing 5 mgm. of the polysaccharide complex per cubic centimeter. Reactions were read at 24 and 48 hours. The results were not in accord with previous immunological observations of similar nature because the non-immunized controls likewise showed cutaneous responses to the injections. This response usually consisted of an erythematous area of ½ to 1½ cm. in diameter with a raised center. One to two weeks after the injection, a round, firm lump ½ cm. in diameter usually formed at the site of injection. This was filled with a white exudate of the consistency of lard surrounded by a well defined wall of fibrous tissue. There was no indication of infection. These sterile abscesses occurred in animals both immunized and untreated.

DISCUSSION

The cross reactions obtained by use of agglutination and precipitation with the anti-bacterial and anti-polysaccharide complex antisera are in accord with the antigenic classification of White (1926), Topley and Wilson (1936) and Kauffmann (1937). The immunological relationships given by *E. typhosa*, *S. enteritidis* and *S. pullorum* would be expected as they share the O antigen IX and the accessory factor XII. Cross reactions in precipitation tests between *E. typhosa* and *S. enteritidis* were obtained also by Furth and Landsteiner (1928), (1929) using anti-bacterial sera and non-antigenic polysaccharide extracts. The cross reactions between *E. typhosa* and *S. paratyphi B* are explained by the common content of the accessory O antigen XII. Furth and Landsteiner (1928), (1929) noted this relationship. Common precipitation and agglutination of *E. typhosa* and *S. paratyphi A* is not accounted for by the presence of a common identified O antigen.

The marked antigenic relationship between *S. paratyphi B* and *S. aertrycke* as indicated by the agglutination and precipitation tests is in conformity with their common possession of

the O antigens I, IV, V and the accessory factor XII. This relationship is reported by Raistrick and Topley (1934) and Boivin and Mesrobeanu (1937) using their antigenic polysaccharide complexes and antisera prepared with them for precipitation tests. Antigens VI and VII shared by *S. paratyphi C* and *S. suipestifer* account for their close antigenic relationship.

The immunizing activity of the polysaccharide extract prepared indicates that it contains all of the factors of the O antigen of the organisms identified in the White-Kauffmann scheme of classification. It is antigenic though free from demonstrable intact protein. Its properties indicate that it is similar or identical to the antigenic extracts prepared by Boivin, Mesrobeanu and Mesrobeanu (1933b), Raistrick and Topley (1934) and Morgan (1937), which are probably polysaccharide-lipoid complexes.

The tendency of the polysaccharide extract on intracutaneous injection in rabbits to form sterile abscesses is of interest. Obviously this action is not related to sensitivity as it occurs in normal as well as in immunized animals. The response which forms a sterile abscess indicates some peculiar property of the extract.

The polysaccharide extract may contain inert materials since White (1929) found that precipitation of the polysaccharide of *S. aertrycke* with 2 volumes of alcohol produced material serologically inert while 4 volumes caused the specific substances to precipitate. However, in the precipitation tests with our polysaccharide extract, all of the material was thrown down by the immune sera for the supernatant was clear.

The authors are indebted to Mr. H. C. Upham for technical aid in this work.

SUMMARY

1. An extract of polysaccharide prepared from mucoid cultures of *Eberthella typhosa, Salmonella paratyphi A, Salmonella paratyphi B, Salmonella paratyphi C, Salmonella aertrycke, Salmonella enteritidis, Salmonella suipestifer* and *Salmonella pullorum* gave cross precipitation tests which parallel the cross agglutination

reactions obtained by use of antibacterial sera for these organisms. These cross reactions conform to the antigenic classification of White and of Kauffmann.

2. These polysaccharide extracts free of intact protein give rise to the formation of precipitins for the polysaccharides and agglutinins for the organisms when used to immunize rabbits.

3. The antisera prepared against the polysaccharide extracts show all of the cross reactions of the antibacterial sera in tests by agglutination and precipitation.

4. The intracutaneous injection of the polysaccharide in normal or immune rabbits leads to the formation of sterile abscesses.

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MENINGITIS CAUSED BY ATYPICAL GRAM-NEGATIVE COCCI

HOBART A. REIMANN, AND RUDOLPH W. KOUCKY

Department of Medicine of the Jefferson Medical College and Hospital and the Pathological Service of the University of Minnesota Hospital

Gram-negative cocci found in samples of purulent spinal fluid during routine laboratory examinations are apt to be reported as meningococci without further confirmation. While the meningococcus no doubt is the most common gram-negative coccus associated with acute meningitis, there are many others which play a rôle, particularly in sporadic cases. Error in diagnosis is also apt to occur in certain cases of meningitis caused by metachromatic cocci or by gram-positive cocci if the Gram stain is improperly applied, and also as a result of the fact that cocci, ordinarily gram-positive, may be decolorized when they are dead or phagocyted. Recognition of these problems and greater care in laboratory diagnosis will prevent wasting antimeningococcus serum in disease not caused by typical meningococci and may help to explain the failure of specific antimeningococcus serum in certain instances.

Branham, Mitchell and Brainin, (1938) recently pointed out the difficulties attending the differentiation of even such well-known bacteria as gonococci from the meningococcus in cases of meningitis. Many of the strains of gonococci which they studied were agglutinated by polyvalent antimeningococcus serum. Sugar fermentation reactions were somewhat more reliable in differentiation but the most fundamental differences appeared in cultural studies pertaining to colony morphology and growth requirements. Even more confusion in diagnosis is apt to arise in dealing with other gram-negative cocci such as *Micrococcus catarrhalis, Micrococcus florens, Micrococcus crassus,*

Micrococcus flavus and other still unnamed varieties, or when multiple infection with two or more strains occurs. German bacteriologists thirty or forty years ago were especially concerned with the problem and many of their publications are referred to in the papers of Forbes (1920) and others. A prolonged controversy arose as to whether the different atypical microörganisms encountered were (a) separate and distinct types of bacteria, (b) variant or mutant forms of the meningococcus, (c) secondary invaders, or (d) contaminants. The problem is illustrated in the following two case reports and studies, the first of which apparently involved an atypical gram-negative coccus; and the second, a type I meningococcus with unusual growth characteristics, together with an atypical staphylococcus.

Accurate knowledge of a complex problem is always desirable.

Case 1 (by courtesy of Dr. I. McQuarrie). A girl, aged 7, suddenly vomited and had diarrhea and headache. She was admitted to the pediatric department of the University of Minnesota Hospital four days later with a diagnosis of meningitis. Rigidity of the neck, Kernig's sign and other disturbed reflexes were noted. A blood culture was negative. The spinal fluid was under normal pressure, but was turbid, contained 68 leukocytes per cu. mm. (56 per cent polymorphonuclear cells) and numerous extracellular and intracellular gram-negative diplococci, which were reported to be meningococci. Antimeningococcus serum was promptly injected intraspinously and subsequent doses were given daily for 6 days. The child improved and her temperature returned to normal about 10 days after admission to the hospital.

Bacteriologic studies. Gram-negative cocci were seen in smears from the spinal fluid before serum injection and on the day after. They were cultivated from the same samples of fluid and also from spinal fluid taken on the fourth day of treatment. Growth was obtained from the spinal fluid only in liver-peptone-broth tubes. The broth became turbid after 24 hours at 37°C. and no sediment or pellicle formed. Subcultures grew on ordinary laboratory media at temperatures between 10° and 37°C., better toward the higher level. On plain agar and blood agar plates the colonies after 24 hours at 25°C. were about a millimeter

in diameter, dull, grayish and translucent.   Several days later,
the colonies measured 3 or 4 millimeters in diameter and opaque
yellowish-gray sectors and wedges formed in many of the colo-
nies.   Subcultures of the translucent portions reproduced trans-
lucent colonies with later opaque sector development.   Subcul-
tures of the opaque area produced similar opaque colonies (fig. 1)
in which translucent sectors and wedges appeared after a few
days.   In one opaque point colony a large translucent wedge
formed and at the edge of the translucent portion an opaque
wedge developed.   The cocci from both forms were identical in
morphology and in biological behavior.   They were usually
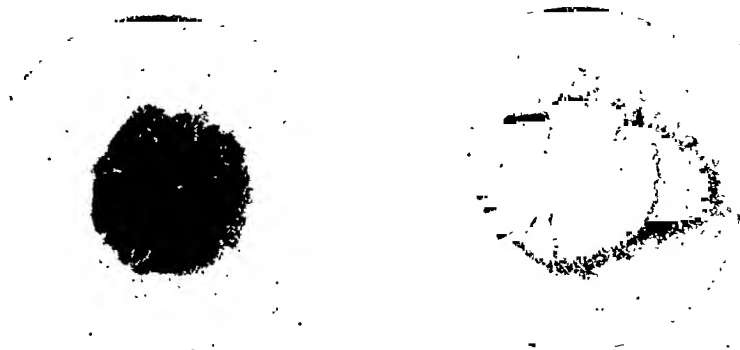
FIG. 1. COLONIES ABOUT TWO WEEKS OLD FROM CULTURE OBTAINED IN CASE 1
   Magnified about × 6.   Several translucent sectors are shown in the opaque
colonies.

gram-negative but, unless thoroughly decolorized, many retained
the purple color, especially in old cultures.   The cocci were
oval or spherical, usually in pairs with adjacent sides flattened,
occasionally single or in clusters.   The long axis of the diplo-
forms was at right angle to the line of union.   There was marked
variation in size.   Cocci from both opaque and translucent colo-
nies caused hemolysis on blood agar, formed an acid coagulum
in milk after 7 days at 37°C, slowly liquefied gelatin, caused no
change in maltose, mannitol, or sucrose broth, but formed acid
after 7 days in glucose and less in lactose broth.   No agglutina-
tion occurred in various types of antimeningococcus serum

obtained from Dr. C. P. Miller of Chicago, from Dr. S. Branham of Washington and from commercial sources. Broth cultures were avirulent when injected intraperitoneally into white mice, guinea pigs and rabbits.

It is obvious that the bacterium is not a meningococcus nor does its behavior coincide in all respects with the criteria laid down for *Micrococcus catarrhalis*, *Micrococcus florens* (Davison, Davison, and Miller, 1917), *Micrococcus crassus* (Elser and Huntoon, 1909), *Micrococcus flavus* and others (Branham, 1930). It has many, but not all of the characteristics of each of the varieties named, but for reasons given in the discussion it does not seem desirable to give the strain a new name.

The second case apparently involves the problem of dual infection and of type transformation which confronted Von Hibler (1907), Stoevesandt (1908), Forbes (1920), Köhlisch, (1915), McDonald (1908), Kempf, Gilman and Zerfas (1933) and others (Sen, 1936).

Case 2. A young man, age 23, was said to have had meningitis following sinusitis in 1932. On April 10, 1936 he noted sore throat and awakened the next day with severe headache followed by vomiting and chills. He was then admitted to the Student Health Service of the University of Minnesota Hospital. His throat was inflamed and a shallow ulcer was seen in the right tonsillar fossa. His neck was slightly rigid; there was general hyperesthesia of the skin, but no disturbance of reflexes until later in the day when Kernig's sign appeared. The leukocytes numbered 27,000 and a gram-positive coccus was cultivated from the blood. The spinal fluid was under 24 mm. of mercury pressure and contained 8000 cells of which 70 per cent were polymorphonuclear. A direct smear of the spinal fluid examined in the Minnesota State Board of Health laboratory showed numerous intracellular gram-negative diplococci. The patient was then given antimeningococcus serum intraspinally and intramuscularly. There was considerable improvement in his condition on the following day, but the neck was still rigid. His temperature declined and became normal after several days.

Bacteriologic studies. As a part of a routine procedure, portions of the same specimen of spinal fluid were sent to the Min-

nesota State Board of Health laboratory and to the University Hospital laboratory. The former reported the presence and subsequent cultivation of typical meningococci and the hospital laboratory reported the presence of gram-positive intracellular diplococci. The gram stain of the latter was retained even when decolorization was carried to the point at which all color was gone from the nuclei of the leukocytes in the smear.

The meningococcus. Because of the different reports from the two laboratories, cultures of the meningococcus first isolated were sent to Dr. C. P. Miller and to Dr. Sara Branham both of whom confirmed its identity as type I meningococcus. The strain was subsequently tested in our laboratory and found to have certain unusual characteristics. The culture from a Loeffler medium slant was seeded in 9 stab punctures in a deep (10 mm. thick) plain agar plate. After 48 hours at room temperature growth appeared at 3 of the 9 points of inoculation. The colonies were translucent and composed mostly of gram-negative diplococci, but, here and there, were found distinctly gram-positive cocci, even after thorough decolorization. Another culture was made in a 100 cc. flask of broth and incubated 6 days at 37°C. Subcultures on plain agar showed two forms of colony, large and small, composed of similar cocci most of which were gram-negative, but a few were gram-positive. Numerous tetrads were present and the cocci were pleomorphic and metachromatic.

The Gram-positive cocci isolated in the hospital laboratory. Gram-positive cocci, which were cultivated from the spinal fluid and from the blood before serum treatment, and from the spinal fluid on the day after, were apparently identical. Subsequent cultures from the blood and spinal fluid were sterile. Fluid from the second spinal fluid specimen was inoculated in numerous stabs in a plain agar plate and growth occurred at each site, the colonies measuring 2 mm. after 6 days at 25°C.

A culture in liver-peptone-broth from the first specimen was plated on plain agar. Three forms of colonies appeared (1) many small translucent ones, (2) several larger, thicker, opaque brownish ones and (3) a few opaque white ones. The appearance

of a variety of colonies was similar to the experience reported in a case of meningitis caused by *Micrococcus tetragenus*, and was regarded as evidence of variation in a single strain of bacteria.   Subcultures from each of the colony types bred true. The brown colonies on occasion developed both translucent and white forms as wedges or daughter colonies.   The cocci isolated from the blood first produced colonies of the white type, and in later subcultures similar translucent, and the brownish, forms appeared.

Cocci from all three colony types were gram-positive and were arranged singly, in pairs, in tetrads and in clusters like staphylococci.   Certain characteristics of the three types are shown in table 1.

TABLE 1

Maltose mannitol lactose glucose sucrose milk gelatin hemolysis

	Maltose	mannitol	lactose	glucose	sucrose	milk	gelatin	hemolysis
White.............	++	−	+	+	+	Ft. acid	Liquefied	±
Brown............	+	−	+	+	+	Acid	Liquefied	+
Translucent.......	+	+	+	+	+	Thick coagulum	Liquefied	++

Tests made by Dr. W. A. Kreidler.

Cocci from all three forms were avirulent for white mice after intraperitoneal inoculation.   Agglutination tests were negative in serum obtained from the patient, but were irregularly positive in low dilutions of several samples of antimeningococcus serum, regardless of source or type.

We believe that the patient had primary meningococcus meningitis and was host to a staphylococcus which became a secondary invader.   The three colony forms were regarded as variants of the strain of staphylococcus in question.   It is too remote a possibility to suggest that the staphylococcus was a mutant form of the meningococcus.   A few experiments were made to determine whether or not growth of meningococci in the liver-peptone-broth used in our laboratory tended to transform them into gram-positive cocci.   Nine strains of meningococci were grown for various periods up to 6 months in liver-peptone-broth.   Transplants were made once or twice a week.

Plate cultures from these tubes at various times revealed the usual type of colony which was smooth and transparent, and two variant forms, one, rough and the other, moist and sticky. No gram-postive cocci were encountered from any of the colonies examined.

It is probable that under ordinary routine diagnostic conditions the gram-positive cocci isolated from the patient's spinal fluid in our laboratory would be disregarded or dismissed as contaminants, yet the presence of identical cocci in the blood and similar experiences of other observers, notably Köhlisch (1915) and Kempf and his associates (1933), show that the occurrence of more than one species of bacteria in cases of meningitis is not uncommon.

DISCUSSION

The problem in both cases appears to involve the question of bacterial variation or type transformation as described previously (Reimann, 1935) in a case of meningitis caused by *Micrococcus tetragenus* in which three colony forms of *Micrococcus tetragenus* were immediately detected and later 13 additional variant forms of the original strain were isolated.   These various types derived from each other differed considerably in several of the biological tests commonly used.   Certain of the differences were as great or greater than those which are customarily used to separate and identify the closely related strains of the *Neisseria* group and the various atypical meningococcus strains (Reimann, 1937). In case 1, the gram-negative bacterium showed two colony forms neither of which had characteristics to identify it with previously established varieties.   It was not determined however, whether they were variant forms or types of certain known varieties or a separate variety.   It is obvious, however, that not all gram-negative cocci found in the spinal fluid in cases of meningitis are meningococci.

The problem in case 2 was still more complicated.   The type I meningococcus recovered grew on plain agar at room temperature, which is at variance with all but a few descriptions of meningococci, and further, many of the cocci retained the

gram stain. The latter observation was similar to that of McDonald (1908) and others, yet Murray (1929) voices the general opinion that meningococci are never gram-positive. Two colony types were observed in old cultures as noted also by Mc-Donald (1908) and Canti (1918).

The gram-positive cocci isolated from the spinal fluid and from the blood, might have been looked upon either as mutation forms of the meningococcus as suggested by Köhlisch (1915), or as staphylococci and secondary invaders. Here again evidence of type transformation was present among the three colony forms derived. There were therefore obtained from the same spinal fluid sample five different colony types, namely, two forms of the meningococcus and three of the staphylococcus. Under the old monomorphic conception of classification these may have been mistaken for five separate strains, or as evidence of contamination, but in reality they represent the variant forms or types of a member of the *Neisseria* group and those of a staphylococcus which we believe was a secondary invader. Similar circumstances may have caused the polemic of thirty years ago about the relationship between the meningococcus, the *Diplococcus crassus* of Jaeger and Von Lingelsheim, and the staphylococcus. Viewed in the light of present knowledge of bacterial variation and multiple infection, the problem still seems complicated but less obscure. On the evidence submitted, one may suggest the following possibilities: first, it would seem that any of a number of bacterial strains ordinarily harbored in the nasopharynx or elsewhere may become invasive and cause meningitis when the resistance of the host is reduced; second, a given strain may be present in several variant forms of type or culture phase (mucoid, smooth and rough), and third, secondary infection with another organism with its variant forms may also be present. This view of the matter reconciles many of the old ideas since it is now known that numerous bacteria may cause meningitis and any given strain may manifest itself in a number of variant forms.

One is inclined to doubt with Hadley (1927) and Sherman (1937) whether it is possible ever to classify bacteria except on

a broad basis. Sugar fermentation, agglutination and other tests have lost much of the importance heretofore attached to them in differentiation. In our own experience many of these tests give inconstant results in the hands of different workers and even for the same person working with the same materials and same technic at different times. Certain types of bacteria indeed seem to have relatively constant characteristics, but the evidence of type and culture phase instability as demonstrated among pneumococci, *Micrococcus tetragenus* and staphylococci suggests that similar variation may occur among other bacterial species. Evidence of type variation among meningococci has already been published (Atkin, 1925; Branham, 1937).

SUMMARY

Two cases of meningitis associated with atypical bacteria were studied. From the first patient (case 1), a gram-negative diplococcus with characteristics different from other classified strains was isolated. The bacterium gave rise to two interchangeable colony forms. From the second case, two species of cocci were obtained; one was a type I meningococcus which had the unusual ability to grow on plain agar at room temperature and had gram-positive elements, the other was a staphylococcus which was represented by three interchangeable colony forms.

These studies suggest that under ordinary laboratory routine examination, atypical colonies may appear on culture plates. Such colonies are usually ignored or dismissed as contaminants, but they may actually be part of the pattern of variation of the bacterium concerned. Recognition of the phenomenon of bacterial variation and type transformation would remove much confusion and would probably reduce the number of separate varieties of bacteria now believed to exist.

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AN IMPROVED METHOD FOR TESTING ANTISEPTIC DUSTING POWDERS

JOHN H. BREWER

Bacteriological Laboratory, Hynson, Westcott and Dunning Company, Baltimore, Maryland

As Garrod (1935) and others have pointed out, there is a definite need to test antiseptics by methods which more closely approximate the actual conditions of use rather than by arbitrary laboratory procedures.

The agar-plate method suggested by Circular 198, United States Department of Agriculture, for testing dusting powders is a very convenient method but should be modified to prevent misleading results and to simulate more closely actual use.

In some recent work with antiseptic dusting powders, it was found that when powders containing some of the essential oils, known to be only slightly bacteriostatic, were placed on agar plates in the amounts shown in the U. S. D. A. Circular 198, a good zone of inhibition would be produced. When amounts actually used in practice were tried, no zone of inhibition was obtained. It was then decided to modify the test in such a way that the amount of dusting powder would be more nearly equivalent to the amount which actually adheres to the skin.

The method found to be most satisfactory is as follows:

Several circular shields are cut from 3 x 5 filing cards. These shields have a 10 mm. square cut from the center and a small tab to facilitate handling. About twenty-five of these shields are placed in a petri dish and sterilized in the hot air oven. The agar plates are made and seeded with *Staphylococcus aureus*, as suggested in the U. S. D. A. circular (0.1 cc. of a twenty-four hour broth culture to 20 cc. of melted agar previously cooled to 42° to 45°C.). Unglazed porcelain-top dishes are used so that
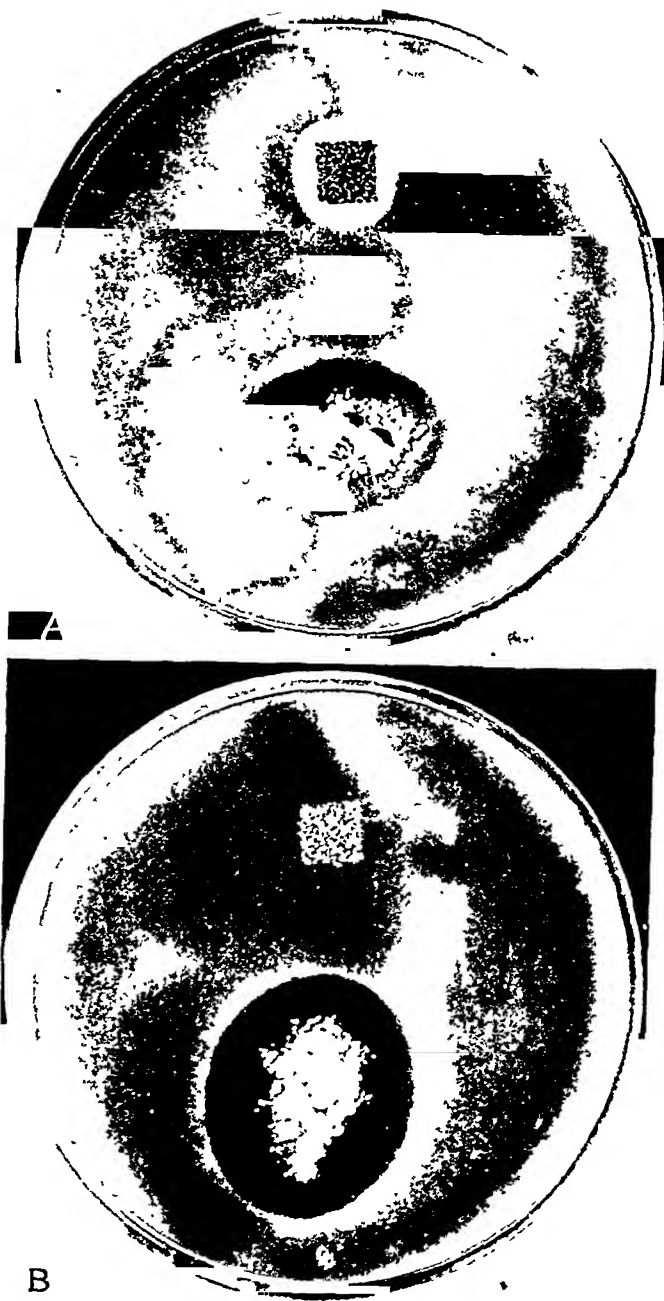
FIG. 1. *A*, plates showing inhibition with small amounts of powder which will adhere to skin; *B*, plates showing inhibition when large amounts are used, but no bacteriostatic action when amounts equivalent to those actually employed on the skin are tested.

there will be no collection of moisture on the surface of the medium. After the agar has hardened, one of the sterile shields is lifted onto the surface by means of the tab which is bent so that it does not touch the agar. About a gram of the dusting powder to be tested is placed on a piece of sterile gauze, approximately 4 inches square (52 mesh Brookside bunting was used), and the corners brought together to form a small bag. The powder is dusted from this bag onto the shield until the 10 mm. square of agar is just covered. The mask is removed, placed in a discard jar, and the petri dish turned upside down to let any excess powder fall off. The lid is replaced and the plate incubated. If desirable at this point, a small spatula of the dusting powder may be placed on the agar opposite the 10 mm. square in order to compare the results with the U. S. D. A. method.

When three of the most popular antiseptic dusting powders on the market and that prepared according to the formula given in the National Formulary for an antiseptic dusting powder were tested by this method, the N. F. powder and only one other gave zones of inhibition when amounts equivalent to those which will adhere to the skin were tested (fig. 1, A). Two of the most popular brands which give good zones of inhibition by the U. S. D. A. method give no zones at all when amounts actually used are tested (fig. 1, B).

SUMMARY

A simple method of testing antiseptic dusting powders which more closely simulates the actual conditions of use has been described. This test requires the additional use of masks made from 3 x 5 filing cards and a small piece of fine mesh gauze.

This test eliminates false results due to concentration of essential oils, standardizes the zone of application of the dusting powder, and thereby unifies the results for comparison.

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THE PATTERN OF DISSOCIATION IN HEMOPHILUS INFLUENZAE

CAROLINE A. CHANDLER, LEROY D. FOTHERGILL AND
JOHN H. DINGLE

WITH THE TECHNICAL ASSITANCE OF MARGARET S. McMILLAN

Department of Bacteriology and Immunology, Harvard University Medical School

In recent communications, Hadley (1937), Dawson (1934) and others have pointed out that most, if not all micro-organisms exhibit a similar dissociative pattern. Dawson (1934, 1938), in studies on the pneumococci and the hemolytic streptococci in particular, has shown that the pattern of variation in these two organisms is strikingly similar. In view of this, both Dawson and Hadley have proposed that a uniform nomenclature for all micro-organisms be adopted to designate the three chief phases of dissociation—i.e., mucoid (M), smooth (S) and rough (R).

In 1931, Pittman described a smooth and a rough form for *Hemophilus influenzae*. Smooth strains were characterized by (a) the appearance of their colonies, which were large, smooth, mucoid, opaque and iridescent in obliquely transmitted light; (b) the fact that the bacteria were capsulated; (c) the production of a soluble specific substance (present in culture filtrates and washings of the bacteria). Rough strains formed colonies which were rough and irregular in outline, smaller in size than the S colonies, less opaque and non-iridescent. The organisms were not capsulated, and none of the R strains produced soluble specific substance. The S strains were found to be more pathogenic for laboratory animals than the R strains.

Huntington (1935), referring to Dawson's classification of the pneumococci, suggested that the same terminology be adopted to designate the dissociative pattern of *H. influenzae*. Accord-

ingly, he applied the term "mucoid" to the strains which produced mucoid, fluorescent colonies, capsulated organisms and soluble specific substance (i.e., Pittman's S). He went no further, however, than stating that the S form was the "ordinary" strain and the R the rough variant.

In previous reports by the authors (Chandler, Fothergill and Dingle, 1937; Fothergill and Chandler, 1936; Fothergill, Dingle and Chandler, 1937) the problem of variation in *H. influenzae* has been discussed, particularly from the point of view of its relationship to morphologic, serologic and pathogenic characteristics of the organism. The terminology previously employed by them to describe the dissociative phases of this organism has not, unfortunately, been of aid in clarifying the confused nomenclature. In the present paper, the problem of variation in *H. influenzae* is reviewed in the light of recent observations on microbic dissociation. The data obtained indicate that the three-phase system of dissociation holds for this organism.

The following section headed *Experimental* is a compilation of the authors' earlier observations, together with additional experimental data hitherto unpublished.

EXPERIMENTAL

Methods

Media: broth. To provide the necessary growth factors for this organism, Filde's peptic digest-blood was added to ordinary infusion broth to a concentration of 5 per cent.

Plates. In order to study colonial morphology and variation, a transparent agar medium was used similar to that described by Levinthal and Fernbach (1922). Streak plates were invariably inoculated from broth cultures in such dilution as to give widely separated colonies. A Zeiss colony microscope was employed in studying colonial morphology. The presence or absence of fluorescence was determined by examining the plates in transmitted, artificial light.

Capsule stain. Several capsule stains were employed, including Hiss', Wright's and Enders'.

Precipitin tests. Precipitin tests were performed according to the technique described in a previous paper (1936).

Virulence determinations. Virulence determinations in mice were

carried out by the mucin method (Fothergill, Dingle and Chandler, 1937a). Henceforth, in this paper, the term "mouse-virulence" implies its determination by this technique.

Cultures. All strains employed were originally isolated from (a) the spinal fluid of patients with meningitis or the blood of patients with bacteremia and (b) the respiratory tract of patients with mild upper respiratory infections. All the rough strains referred to in this paper were derived from Type *b* mucoid strains by prolonged growth on artificial media or repeated transfer in Type *b* immune serum broth.

Characteristics of strains isolated from the spinal fluid of patients with H. influenzae meningitis and the blood of patients with H. influenzae bacteremia

Colonial morphology. Large, mucoid, smooth, convex, glistening, opaque, markedly fluorescent in transmitted light.

Morphology of organisms. Capsulated, coccobacilli or short rods, uniform in size and staining properties.[1]

Growth in broth. Diffuse.

Type-specificity. An essentially homogeneous group, serologically, producing soluble specific substance.

Virulence. Virulent for man (probably for young children and infants only (Fothergill and Wright, 1933)). Virulent for mice by mucin technique.

Stability. Very unstable on primary isolation. Plates of first subculture frequently show, in addition to the mucoid colonies, a few variants of the rough type. Once growth is established in a favorable medium, however, strains become very stable and produce only mucoid, fluorescent colonies. After varying lengths of time (6 months to 2 years) variants begin to appear.

Characteristics of strains isolated from the upper respiratory tract in non-specific respiratory infections

A. Predominant strains

Colonial morphology. Small, smooth, convex, slightly conical, bluish, translucent, non-fluorescent in transmitted light.

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[1] Smears made directly from the spinal fluid frequently showed very pleomorphic forms, but these were replaced by non-pleomorphic coccobacilli after subculture.

Morphology of organisms. Non-capsulated coccobacilli or short rods, uniform in size and staining properties; larger rods occasionally present.

Growth in broth. Diffuse.

Type specificity. Serologically heterogenous; specific soluble substance not present.

Virulence. May or may not be virulent for man; slightly virulent to avirulent for mice by mucin technique.

Stability. Relatively stable when freshly isolated, but some strains after prolonged growth on artificial media produce colonies which are rough. Spontaneous reversion to the mucoid form has not been observed.

B. Other strains

Approximately 80 to 90 per cent of strains isolated from the respiratory tract in mild respiratory infections exhibit the characteristics listed above. Aside from occasional *"intermediate"* forms, the rest of the strains isolated from this source fall into (a) the mucoid group and are fluorescent, capsulated and type-specific (a, b, c, d, e, or f) (Pittman, 1931; Platt, 1937), (b) the rough group and are non-fluorescent, non-capsulated, non-type-specific and avirulent for mice.

Characteristics of strains dissociated by means of prolonged growth on artificial media or by repeated transfer in broth containing type specific immune serum

Colonial morphology. Fairly large, rough, slightly convex to flat, edge irregular to serrated, translucent or slightly opaque, bluish, non-fluorescent in transmitted light.

Morphology of organisms. Non-capsulated, long rods, clubs, filaments, very irregular in size, shape and staining; markedly pleomorphic.

Growth in broth. Granular or flocculent.

Type specificity. Serologically heterogenous; specific soluble substance not present.

Virulence. Completely avirulent for mice by mucin technique.

Stability. When fully dissociated, the rough form is very

stable over long periods of time. In intermediate stages of dissociation, the morphology is more variable. A strain which is in the process of changing from S → R may suddenly produce colonies which are much smoother than the colonies of the previous plating. With continued transfer in serum broth, however, the culture eventually goes over to the R phase. Concomitantly with this S → R shift, there is a more or less progressive loss of type-specificity, but in some instances a strain may retain slight traces of SSS after its colonial form has become distinctly rough. On the other hand, type-specificity may be lost at a stage when the strain is in an intermediate phase morphologically.

After prolonged cultivation on chocolate agar, many rough strains spontaneously give rise to a number of variants, in a few instances a smoother component than the original form, but most frequently one or more very rough or "degraded" forms. One such strain, a Type *b* when first isolated from a blood culture, produced (after artificial dissociation) a single type of rough colony which had all the characteristics of the rough phase. When plated out after two years' cultivation on artificial media, this strain produced three kinds of colonies. Since at least one of these variants has not, to the authors' knowledge, been described[2] before, a detailed description of the characteristics of all three variants is listed in table 1. For purposes of comparison, the characteristics of the original and the converted rough forms of the strain are also given in the table.

Reversibility of the R to the S or the M phase

The fact that smooth colonies may appear spontaneously in a culture which according to all criteria is in the rough phase has been stated previously. It has also been stated, however, that the spontaneous reversion of a smooth to a mucoid form has not been observed; nor has it been possible to bring about

[2] None of the photographs in Holster's (1931) excellent paper on variation in *H. influenzae* resembled this variant closely enough to permit its identification with any of the variants described.

TABLE 1

	ORIGINAL	CONVERTED ROUGH	VARIANT A	VARIANT B	VARIANT O (NEW)
Colonial morphology	Large, smooth, convex, opaque. (Fluorescence *not recorded*)	Medium size, very rough surface, edge irregular, *bluish translucent* non-fluorescent	Medium size, moderately rough surface, edge regular, *bluish opaque*, non-fluorescent	Very small, smooth, convex round, edge regular, *translucent non*-fluorescent	Tiny (just visible to the naked eye), very flat, very *rough*, *very ir*-regular edge, dull, transparent, non-fluorescent
Morphology of organisms	Short rods and coccobacilli; quite uniform in size and shape	Thick club forms, rods of all sizes, and filaments; marked pleomorphism	Fat coccobacilli, clubforms, few filaments; marked pleomorphism	Coccobacilli, club forms long rods; marked pleomorphism	All long, thin tangled filaments
Precipitin titre (vs type *b* antiserum)	1:8	0	1:2	0	0
Agglutinin titre (vs type *b* antiserum)	1:1280	0	1:640	1:640	1:640
Mouse virulence (in M.L.D.'s)	*	†	5,000,000		200,000,000

* The virulence of 11 similar strains determined at a later date varied from 30,000 to 1,000,000 M.L.D.'s.
† The virulence of 12 similar strains determined at a later date varied from 2 million to 7 billion M.L.D.'s.

this change by cultural manipulation. In an effort to determine whether or not the R → S → M transformation could be accomplished by animal inoculation, the following experiments were carried out:

a. Repeated mouse passage by the intraperitoneal route of a rough strain (in mucin) isolated from a case of meningitis secondary to trauma,

b. Repeated mouse passage by the intraperitoneal route of a rough strain (in mucin) derived from a mucoid, meningeal Type *b* strain,

c. Combined intraperitoneal and subcutaneous injection of a rough strain, originally isolated from the respiratory tract,

d. Combined intraperitoneal and subcutaneous injection of a mucoid, Type *b* filtrate, together with a rough strain, originally isolated from the respiratory tract,

e. Combined intraperitoneal and subcutaneous injection of a mucoid Type *b* vaccine, together with a rough strain, originally isolated from the respiratory tract.

In no instance was the transformation R → M accomplished by any of the above methods. On the other hand, the R → S conversion was brought about by every method but the last. In most instances, however, the S form did not maintain its stability, and after several transfers on artificial media reverted to the R form.

It is interesting to note that the R → S conversion took place very gradually in experiments (a) and (b), whereas in experiment (c) the change occurred abruptly. The progressive change in colonial form from R → S in (a) and (b) was accompanied by the appearance of "swollen" or "globoid" forms in smears of the peritoneal exudates. After twenty to thirty mouse passages, the "swollen" forms were replaced by coccobacilli, uniform in size and staining properties. In experiment (c), in which the R → S change occurred abruptly, the S colonies appeared on plates streaked with pus from the subcutaneous abscess. Smears of the same exudate showed no "swollen" forms, but only coccobacilli which were uniform in size and staining properties.

A summary of the characteristics of the three phases of dissociation in *H. influenzae* is given in table 2.

TABLE 2

Variation in Hemophilus influenzae

	Mucoid (fluorescent)	Smooth (non-fluorescent)	Rough (non-fluorescent)
Morphology of colonies			
Morphology of organisms	Coccobacilli	Coccobacilli or short rods; pleomorphism slight	Long rods, clubs, long tangled filaments, pleomorphism marked
Capsules . .	+	0	0
Growth in peptic-digest broth	Diffuse	Diffuse	Flocculent
Type specificity	+	0	0
Virulence:			
Human	+ or ±	±	0
Mouse	+ or 0	± or 0	0

DISCUSSION

Morphologically and culturally, the mucoid, smooth and rough phases of *H. influenzae* correspond very closely to the mucoid, smooth and rough phases of other organisms. Mucoid strains, which are highly virulent for man, produce a soluble polysaccharide and form a homogeneous group serologically (Type *b*). Mucoid strains less frequently associated with infection in man also produce a soluble polysaccharide, but in general fall into different serological types (a, c, d, e, f).

Smooth strains are characterized by their morphology, lack of type specificity and relative avirulence for man and animal. It should be emphasized that the "S" in the old classification is the M or mucoid as here classified. The S type of the present terminology is found in non-specific respiratory infections, sinusitis, bronchiectasis, etc., and has little if any pathogenic significance.

Rough strains are occasionally isolated directly from man, but are probably not associated with infection. Fully dissociated rough strains are, in general, only found in old, degraded stock cultures, or are derived from mucoid strains by experi-

mental methods: They are characterized by their morphology, lack of type specificity and avirulence for man and animal. They can be distinguished from smooth strains only by the appearance of their colonies and by the morphology of the individual bacteria.

It should be emphasized at this point that, whereas each variant phase can usually be distinguished by its serologic and pathogenic, as well as by its morphologic properties, the basis of differentiation ultimately depends on morphology.   Dawson has pointed out that although all hemolytic streptococci which are virulent for mice are mucoid, capsulated and type-specific, not all mucoid strains are virulent for mice.   In the authors' experience, this has also been true of *H. influenzae*.   Strains which are highly virulent for mice are mucoid, fluorescent, capsulated and type-specific, but not all mucoid strains are mouse-virulent.   Mucoid strains of Types *a* and *d*, for example, were found to be relatively avirulent for mice, although morphologically they were in the mucoid phase.

The M → S → R transformation may occur spontaneously in old stock cultures or can be brought about by ageing or by repeated transfer in homologous, type-specific, immune serume broth.   The R → S conversion may also occur spontaneously, but probably happens rarely.   R → S conversion can be induced by animal inoculation.   This transformation may be brought about gradually by repeated mouse-passage intraperitoneally, or abruptly by means of combined subcutaneous and intraperitoneal inoculation.   In the authors' experience, it has been impossible to effect the transformation R → M or S → M by animal inoculation, either by the intraperitoneal or subcutaneous routes.

Pittman succeeded in converting one R strain to the "S" (M) form by repeated growth in anti-R serum broth and by animal inoculation.   The fact that this particular rough strain had been derived from a "smooth" (mucoid) strain only a short time before, suggests the possibility that the R variant might not have been completely converted to the R phase and therefore still retained some mucoid elements.   This possibility is also suggested by the fact that Todd and Lancefield (1928) in their early studies on the dissociation of the hemolytic streptococcus

found that it was impossible to transform the glossy (smooth) into the matt-virulent (mucoid) form unless the glossy culture retained a small amount of type-specific substance.

Kun and Fenyvessy (1932) also had difficulty in effecting the R → S conversion in *H. influenzae*. They found, for example, that it was impossible to produce a stable S form from an R strain either by cultural manipulation or by intraperitoneal inoculation. The S strains so derived rapidly reverted to the R phase after a few transfers on chocolate agar. By subcutaneous injection, however, they were able to convert rough, "filament forming" strains into smooth "coccobacilli forming" strains which retained their smooth characteristics on artificial media. The increase in rat virulence and the changes in morphology exhibited by these derived strains indicate that transformation from the rough to the smooth phase took place. There is no evidence to indicate, however, that conversion to the mucoid phase was accomplished.

From the present observations and those of other investigators, it therefore appears that *H. influenzae* exhibits a three-phase dissociative pattern, namely, the mucoid, the smooth and the rough. The M → S → R transformation can be readily accomplished, whereas the R → S conversion can probably be obtained only by animal inoculation. The R → M transformation cannot be brought about by the techniques in use at this time.

SUMMARY

1. The mucoid, smooth and rough phases of *Hemophilus influenzae* are described.

2. The interconvertibility of the three dissociative phases is discussed.

3. The pattern of variation in *Hemophilus influenzae* corresponds to that of other micro-organisms.

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Fig. 1. Mucoid Colonies
Magnification approximately 10 ✕
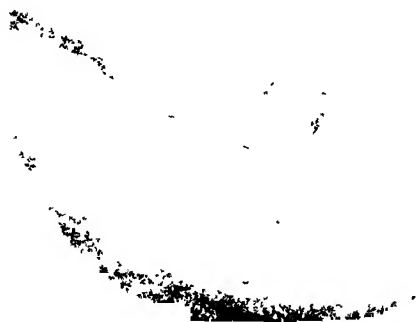
Fig. 2. A Smooth Colony
Magnification approximately 10 ✕
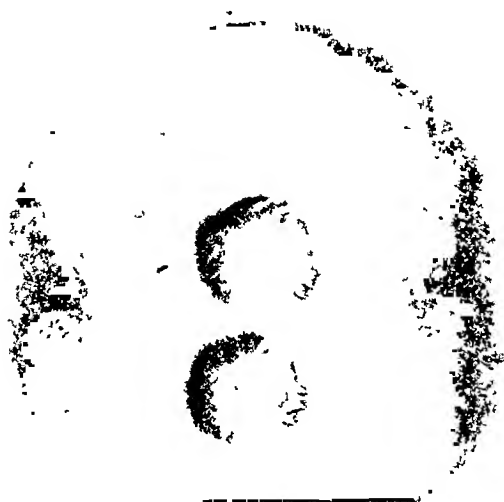
FIG. 3. ROUGH COLONIES
Magnification approximately 10 ×

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THE NUTRITIONAL REQUIREMENTS OF CLOSTRIDIUM PARABOTULINUM, A

S. S. ELBERG AND K. F. MEYER

George Williams Hooper Foundation and Department of Bacteriology, Medical School, San Francisco, California

This investigation was undertaken with the purpose of developing a synthetic medium for *Clostridium parabotulinum, type A,* in order to study certain phases of the toxin-producing mechanism.

The results mainly confirmed the findings of Fildes and his coworkers (1933) with respect to the essential nature of certain substances in yeast and pregnancy urines. In addition, certain evidence is presented which indicates that other substances besides the "sporogenes vitamin" are required for growth of *C. parabotulinum* in amino acid mixtures. The nature of these materials will be the subject of subsequent notes.

HISTORICAL INTRODUCTION

Studies on the simplification of media for the cultivation of *Clostridium botulinum* have not often been the primary purpose of researches on this organism. In fact, it is only in recent years that synthetic media have been attained for some members of the obligate anaerobes, notably *Clostridium sporogenes.*

That the complexity of the nutritional requirements of *C. botulinum* and the need for accessory growth factors were realized is stated in a paper by Wagner, Meyer and Dozier (1925). Hosoya and Kishino (1925) demonstrated the importance of the sulfhydryl group in the growth requirements of *C. botulinum* and found that cysteine adequately supplemented deficient tryptic digests of gelatin. Along the same lines, Quastel and Stephenson (1926) found that the lack of reduced sulfur com-

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pounds was the limiting factor in the inadequacy of acid and tryptic digests of gelatin, although similar digests of casein did support growth.

Anderson (1928) then attempted unsuccessfully to simplify the routine media for *C. botulinum* by using nucleic acids, ammonium compounds, and mixtures of many amino acids as sources of nitrogen.

In a specific attempt to determine the essential amino acids for growth and toxin production by *C. botulinum,* Burrows (1932) found that acid hydrolysates of casein, if supplemented with tryptophane, would support growth of A and B strains. Such hydrolysates were rendered unfit, by treatment with Norite or by filtration through Seitz pads. Burrows (1933) then reported the successful substitution of an amino acid mixture for the protein hydrolysate and stated that cystine, proline, and leucine were essential acids for *C. botulinum.*

A very intensive study on the nutritional requirements of *C. sporogenes* was summarized in a report by Knight and Fildes (1933) in which was detailed the preparation and proof of the essential nature of a substance in yeast and pregnancy urines. Furthermore tryptophane was found to be an essential amino acid for growth of *C. sporogenes.* These authors also determined that the "sporogenes vitamin" and tryptophane were essential for the growth of *C. botulinum.*

Burrows (1934) reinvestigated the tryptophane requirements of *C. botulinum* and found that this organism differed from *C. sporogenes* both in the tryptophane and "sporogenes vitamin" requirements.

Fildes (1935) returned to the study of the requirements of *C. botulinum,* confirmed his former work and reaffirmed the belief that the difficulty lay in the use by Burrows of impure preparations of amino acids containing tryptophane as an impurity.

Fildes and Richardson (1935) working with *C. sporogenes* finally succeeded in replacing the gelatin hydrolysate by a known mixture of amino acids, most of which had been synthesized, and all of which had been recrystallized several times. They concluded that tryptophane, leucine, phenylalanine, tyrosine and

arginine were indispensable amino acids, while for adequate growth and maintenance of the cultures valine, cystine, methionine, and histidine were required.

Stickland (1934, 1935a, 1935b) cleared up the problem of the source of energy for the organism growing in a medium composed chiefly of amino acids by showing that *C. sporogenes* is able to activate certain pairs of amino acids, one being oxidized, the other reduced.

As to the nature of the "sporogenes vitamin" Pappenheimer (1935) found that it was an acid (pK^1 4.7), whose empirical formula was $C_{11}H_{14}O_4$, that it was most stable as the methyl ester, and that it contained one double bond and one hydroxyl group.

Following this there appeared a paper by Hosoya, Kuwashima, Kayo, Oda, and Kagabe (1936) on a factor essential for "pathogenic bacteria." The source of their active preparation was an "aqueous extract of scomber." In amounts of 5×10^{-6} gamma per 10 ml. gelatin hydrolysate basal medium, growth of *C. botulinum* occurred. The paper deals mainly with the preparative aspects, while information concerning the methods of assay or nature of the material is lacking.

METHODS

Media

(a) Isolation of colonies and purification of strains from stock cultures were effected on blood plates prepared as recommended by Schoenholz (1928).

For carrying stock cultures a beef-heart medium was employed in which Neopeptone was used as the principal nitrogenous ingredient. The inclusion of minced pieces of beef heart at the bottom of the tubes made the use of vaseline seals unnecessary.

For the production of spores a casein-digest medium recommended by Sommer (1930) was used.

A medium which was found to initiate excellent growth, toxin production and enzyme formation contained a filtered infusion of beef heart to which were added 1 per cent glucose, 0.5 per

cent monobasic potassium phosphate and 4 per cent Difco proteose peptone. The final pH was adjusted to 7.4. Sterile petrolatum served as an efficient seal.

Inoculum

Several liters of spores of a carefully purified strain of *C. parabotulinum, type A,* were washed five times in sterile M/15 phosphate buffer, pH 7.0 and eventually suspended in sterile double-distilled water. After the purity and viability of the spores were tested, the suspension was heated at 80°C. for forty-five minutes, cooled to room temperature and aseptically removed in 25 cc. lots to a Flosdorf-Mudd "Lyophile" apparatus (1935). The spores were rapidly desiccated from the frozen state under high vaccum and came out of the apparatus in the form of a white powder, perfectly viable and toxigenic. They were stored in small vials and checked for purity, viability and toxigenicity at monthly intervals.

Preparation of protein hydrolysates

Gelatin and casein, having been shown to support growth of *C. botulinum* under specified conditions, were selected to supply the principal nitrogenous substances in the media to be simplified.

(a) Nelson's photographic gelatin was prepared in the hydrolyzed form as recommended by Fildes (1935).

(b) Washed isoelectric gelatin (Peter Cooper) was prepared according to the method of Northrop and Kunitz (1928) and hydrolyzed by refluxing eight hours with five times its weight of sulfuric acid in 30 per cent solution. The acid was exactly removed with baryta, and the filtrate concentrated *in vacuo* to a thick syrup.

(c) Unwashed Peter Cooper gelatin was hydrolyzed in the same way as (b) and worked up to a syrup.

(d) Casein (Schering-Kahlbaum, according to Hammarsten) was refluxed thirty-six hours with 30 per cent sulfuric and worked up to a syrup as in the previous cases.

"Vitamin" preparation

In the main the original procedure used to prepare the "sporogenes vitamin" followed that of Knight and Fildes (1935a). The active preparation from brewer's yeast was such that 1×10^{-6} gram activated 10 ml. of a deficient medium. The final product was diluted so that 0.1 cc. contained 1×10^{-6} gram of dry material.

EXPERIMENTAL

The medium upon which it was desired to improve was that proposed by Fildes (1935) for *C. botulinum*. It was made up as follows:

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Sodium citrate...........................................3.0  grams
Monobasic potassium phosphate...........................4.5  grams
Water...................................................500   cc.
1N Sodium Hydroxide.....................................26    cc.
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Dissolve and add:
```
l-cystine...............................................0.05 grams
l-tyrosine..............................................0.05 grams
dl-valine...............................................0.10 grams
Acid hydrolyzed protein (5 per cent solution)...........50    cc.
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Dissolve; dilute to 600 cc.; pH to 7.4; tube in 6 cc. amounts; autoclave at 121°C. for 20 minutes.
Before the inoculum was added, all tubes received the following:
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Magnesium sulfate (0.4 per cent solution)..................0.1  cc.
Tryptophane (0.2 per cent solution).......................0.1  cc.
Sporogenes vitamin........................................0.1  cc.
1N Sodium Hydroxide.......................................0.25 cc.
Thioglycollic acid (1 per cent in 1N hydrochloric acid)...0.20 cc.
Water to..................................................9.0  cc.
Inoculum (0.01 mgm. dried spores in 100 cc.)..............1.0  cc.
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All of the above components were sterilized in the autoclave. The amino acids and salts were purified by recrystallization 3–5 times before use. The tubes were plugged with cotton wrapped in washed gauze. The thioglycollic acid was used as purchased. The acid-hydrolyzed protein was diluted so that it was 5 per cent based on the weight of the dry protein used. All incubations were carried out in a phosphorus jar at 37°C.

Experiment 1

The first few experiments on this type of medium were entirely confirmatory in nature, namely, to determine for the strain of *C. parabotulinum* selected the necessity for tryptophane and the "sporogenes vitamin" as previously reported (Burrows, 1932, 1933; Fildes, 1935).

The results indicated that *C. parabotulinum* did require these two components in the hydrolysate medium before any growth would occur. Toxin was produced in ninety-six hours following noticeable autolysis. The occurrence of lysis around the seventy-second hour of incubation was a regular sequel to growth in this type of medium and decreased the usefulness of the medium.

TABLE 1

Growth-supporting ability of protein hydrolysate (medium used as on page 5 but without added cystine, tyrosine and valine)

TUBE	INCUBATION PERIOD		TOXIN 96 HOURS
	24 hours	48 hours	
1 Basal medium + Nelson photographic gelatin hydrolysate	+++	++++	+
2 Basal medium + Peter Cooper gelatin hydrolysate..	+++	++++	+
3 Basal medium + Isoelectric gelatin hydrolysate...	+++	++++	+
4 Basal medium + Kahlbaum casein hydrolysate....	+++	++++	+
5 Basal medium + Egg albumin hydrolysate.......	++	++++	+
6 Basal medium + Water to volume..............	0	0	0

Experiment 2

A comparison between the efficacy of acid hydrolysates of gelatin, casein and egg albumen indicated that there was little difference between the abilities of these proteins to support growth in spite of the differences in their amino acid contents.

The slight inferiority of egg albumin was rechecked and confirmed. Efforts to base this on the serine and oxyproline deficiencies of egg albumin by adding these amino acids in amounts equivalent to their respective concentrations in gelatin and casein did not improve the ability of egg albumin to support

growth. Since casein and gelatin supported growth in spite of the many differences in amino acid content (glycine, valine and tyrosine, particularly), it was decided to determine the nature of the substances present in the isoelectric gelatin which was suitable for the growth of the organisms.

Experiment 3.   Fractionation of gelatin hydrolysate

The method which gave the best fractionation of the acid hydrolysate of isoelectric gelatin was one involving the formation of the copper salts of the amino acids and the subsequent separation of these salts based on their solubilities in methyl alcohol and water (Towne, 1928, 1936).

Three fractions were separated which were (I) the alcohol-soluble water-soluble group including proline, hydroxyproline, isoleucine, valine and the "hydroxyvaline" of Schryver, (II) the alcohol-insoluble water-soluble group including glycine, serine, alanine, arginine, histidine, lysine and pyrrolidone compounds, (III) the alcohol-insoluble water-insoluble group which includes leucine, phenylalanine, tyrosine and cystine.

The fractions were tested for their growth supporting ability in a deficient medium composed of tryptophane, sporogenes vitamin, thioglycollic acid, magnesium sulfate, and sodium hydroxide at pH 7.4. (The amount of each component is the same as that used in the other tests and is summarized above.)

The results indicate that the alcohol-insoluble water-soluble fraction of amino acids is sufficient to support growth of *C. botulinum*. Apparently, from a mixture containing glycine, serine, alanine, arginine, lysine, and histidine, supplemented with tryptophane and sporogenes vitamin, energy and structural components were minimally satisfied.

The addition of fraction I to II did not materially increase the amount of growth nor in fact did it stimulate an earlier germination of the spores. The increase in the amount and the earlier germination in the presence of fractions II + III and I + II + III was paralleled by the same increase when the amount of II in the medium was doubled or tripled.

Although there are several ways in which these results can be

explained, the most attractive approach is by way of the Stickland reaction (1934), in which the source of the energy available to the organisms growing in such a medium is based on reactions between pairs of amino acid molecules.

Fraction II contains alanine which has been shown to be activated as a hydrogen donator by *C. sporogenes*, (Stickland, 1934) plus glycine and serine, the reducible components in the Stickland reaction. This might be one system open to *C. botulinum*. Furthermore, when Fraction III was added to II an initially larger amount of growth appeared which did not

TABLE 2

Effect of gelatin hydrolysate fractions on growth of C. parabotulinum

TUBE	24 HOURS	42 HOURS	96 HOURS
1 Medium + 0.25 cc. Fraction I.................	0	0	0
2 Medium + 0.25 cc. Fraction II................	++	+++	++
3 Medium + 0.25 cc. Fraction III..............	0	0	0
4 Medium + 0.50 cc. I.........................	0	0	0
5 Medium + 0.50 cc. II........................	+++	++++	+++
6 Medium + 0.50 cc. III.......................	0	0	0
7 Medium + 0.25 cc. I + 0.25 cc. II...........	++	+++	++
8 Medium + 0.25 cc. I + 0.25 cc. III..........	+	+	+
9 Medium + 0.25 cc. II + 0.25 cc. III.........	+++	++++	+++
10 Medium + 0.25 cc. I + 0.25 cc. II + 0.25 cc. III....	+++	++++	+++
11 Medium + 0.75 cc. I.........................	0	0	0
12 Medium + 0.75 cc. II........................	+++	++++	+++
13 Medium + 0.75 cc. III.......................	0	0	0
14 Medium + 0.50 cc. water.....................	0	0	0

materially increase over that finally obtained with II alone. Fraction III contained leucine and tryosine among other acids from the hydrolysate. Stickland (1934) and Knight and Fildes (1935) reported that, for *C. sporogenes*, leucine is a more readily available oxidizable component than alanine. Fraction III, supplying as it does acids which may be more available in the case of *C. sporogenes*, may act similarly in the case of *C. botulinum*. It remains to test the various amino acids which can be activated by *C. botulinum* to act as donators and acceptors of hydrogen, before carrying over to this organism explanations which may apply only to *C. sporogenes*.

It is also realized that the fractionation of protein hydrolysates by the copper salt method must involve a certain lapping over of fractional components because of faulty technique. If this occurs the magnitude of the concentrations of an amino acid for example may well be within the range of growth-supporting function as demonstrated by Fildes (1935). This would also explain the growth obtained with Fraction II alone.

The amino acids known to be in the various fractions of the protein hydrolysate were substituted for these fractions and tested with a new "sporogenes vitamin" preparation.

The new batch of "sporogenes vitamin" was prepared as previously but with the alteration from the method of Knight and Fildes (1933) that 0.4 per cent hydrochloric acid in methyl alcohol was used as the initial extracting solvent. The extractions were carried out for four days, using fresh changes of solvent, after which the alcohol fractions were evaporated *in vacuo* to dryness, the residue dissolved in hot water, boiled a few minutes and centrifuged. After the supernatant fluid was again concentrated *in vacuo* to a small volume, the steps followed those of Knight and Fildes.

About two hundred milligrams of residual material were obtained from the yeast of which the potency tests in gelatin hydrolysate basal medium revealed that 3.3×10^{-8} gram activated 1 ml. of the deficient medium (detailed on page 433).

Experiment 4

Several different lots of amino acid mixtures were prepared. Lot 1 was composed only of the amino acids known to be contained in the "Towne Fractions II and III." The amounts of each acid in this lot were based on the best available analyses of the amino acids in gelatin. This was further corrected for the amount of hydrolysate, or original protein hydrolysed, which had been shown to support growth.

Lot 2 was more inclusive, containing all the amino acids thought to be in gelatin, and again the amounts of each were based on the reported percentages in gelatin (Bodansky, 1931).

Lot 3 was the mixture developed by Fildes and Richardson (1935) for the growth of *C. sporogenes.*

A brief summary of the mixtures is presented in table 3.

Of those which were not synthetic, the naturally occurring form, purified by recrystallization was selected.

The amino acids were dissolved and added to the following basal mixture:

```
Monobasic potassium phosphate............................4.5 grams
1N Sodium Hydroxide......................................25   cc.
Water...................................................500   cc.
Adjusted to pH 7.4, diluted to 600 cc., tubed in 6 cc. amounts
    and autoclaved
```

Each tube received the necessary amounts of magnesium sulfate, thioglycollic acid, and the test amount of vitamin preparation previously adjusted to pH 7.4 with 1N sodium hydroxide. Total volume was brought to 9 cc. The inoculum, 1 cc. of a spore suspension, brought the volume to 10 cc.

These media were tested with both available preparations of sporogenes vitamin, the old one, now deteriorating in potency towards gelatin hydrolysate media, and the fresh preparation.

A summary of the comparative activities of the two preparations in the various media is tabulated in table 4.

(1) It will be seen that the old preparation of sporogenes vitamin was completely inactive towards the amino mixtures, whereas the fresh preparation supported a moderate amount of growth in this type of medium. It is also evident in the table above that the old preparation was less effective in the protein hydrolysate medium.

(2) The second point indicated is the loss of activity of the new "vitamin" preparation towards the amino acid mixtures, after ageing about a week.

This loss of potency was by no means an "all or none affair" but was a gradual loss, demonstrable by increasing amounts of preparation required for equivalent turbidities.

(3) The third lot of amino acids was far superior to the other two batches, indicating a striking similarity in growth requirements between C. sporogenes and C. botulinum.

(4) It was impossible to replace the "Towne Fractions II and

III" by the amino acids known to be contained therein, suggesting the presence of other substances or the overlapping of the fractions.

TABLE 3

AMINO ACID	PER CENT IN GELATIN (BODANSKY)	GRAMS PER LITER		
		Lot 1	Lot 2	Lot 3
Glycine.....................	25.4	0.029	0.029	0.2
s-alanine....................	8.7	0.085	0.085	0.12
s-leucine....................	7.1	0.624	0.624	0.17
l-proline....................	9.5		0.836	0.15
l-oxyproline.................	14.1		1.24	
s-phenylalanine.............	1.4	0.123	0.123	0.08
s-glutamic..................	5.8		0.51	
s-aspartic...................	3.4		0.308	0.18
s-serine.....................	0.4		0.035	0.14
l-tyrosine...................	0.01	0.0008	0.0008	0.05
l-histidine..................	0.9	0.0792	0.0792	0.05
d-arginine...................	8.2	0.76	0.76	0.05
s-lysine.....................	5.9	0.51	0.51	0.09
l-tryptophane...............	0.0	0.02	0.02	0.02
l-cystine....................	0.2			0.06
s-valine.....................				0.15
s-methionine................				0.07

The acids marked "s-" were synthetic preparations obtained principally from Eastman Co., and Amino acid Mfgrs., U. C. L. A.

TABLE 4

Effect of sporogenes vitamin preparations

DAILY TEST	GELATIN HYDROLYSATE		AMINO ACID LOTS		
	Growth	Toxin	Number 1	Number 2	Number 3
Vitamin (prepared July, 1936)........	++	+	0	0	0
Vitamin (prepared July, 1937)........	++++++	+++	0	++	+++
After one week:					
Vitamin (July, 1936)...............	++	+	0	0	0
Vitamin (July, 1937)...............	++++++	+++	0	+	+

(5) Toxin was demonstrated in amino acid mixtures supporting growth, but was not particularly potent. One-half cubic centimeter of supernatant fluid from the cultures usually killed

unprotected mice within 36–48 hours, indicating a rather weak toxin. However, this comparison must be qualified because of the fact that growth in the amino acid mixtures, although very apparent, was nevertheless inferior.

One conceivable explanation of the results appeared to involve another essential substance present in gelatin and casein acid hydrolysates additive in effect to the yeast preparation. When the organisms were forced to use purified amino acids as their main food source, they required preformed a larger minimum amount of the "sporogenes vitamin," which could be supplied to them in workable volumes of fresh yeast preparations. Assuming that ageing and subsequent deterioration of the "vitamin" resulted in less than the critical amount being available, the organisms would then find it impossible to grow in such synthetic media. In the gelatin hydrolysate, however, enough "accessory factor" might be present to replace the material lost by ageing of the yeast preparation. A statement by Fildes (1935) that gelatin contains minute amounts of "sporogenes vitamin" seemed to support this hypothesis.

If some accessory factor is present in gelatin hydrolysates, sufficient in the presence of yeast preparation to supplement a deficient medium, the addition of small amounts of gelatin hydrolysate plus the yeast factor to amino acid mixtures should raise the concentration of essential material above the critical level and growth should occur.

When amounts of gelatin hydrolysate, sufficient in themselves to support merely a swirl of growth, were added to the amino acid mixtures, containing yeast preparation formerly sufficient to support growth, no additional growth resulted. When the concentration of hydrolysate was stepped up growth was visible, but the control tubes showed that this growth was due entirely to the hydrolysate and was independent of the amino acid base.

The following fractionations of the gelatin hydrolysate were attempted in order to produce a fraction supplementary only in activity and insufficient in itself to support growth.

1. Alcohol extraction

Ninety-five per cent alcohol was added to a portion of the hydrolysate until the precipitate no longer disappeared on heating. A minimum amount of water was added to dissolve the precipitate and the mixture placed in the ice box. After it had settled the precipitate was filtered and washed with alcohol. It was then dried and taken up in distilled water. The original filtrate containing the alcohol-soluble fraction was concentrated *in vacuo* and taken up in water. Growth tests in a deficient medium (page 433) indicated that the materials for growth were concentrated in the alcohol-soluble fraction since this fraction supported growth. The alcohol-insoluble fraction which did not support growth was also non-supplementary to the deteriorated sporogenes vitamin in the amino acid mixture number three.

2. Alcohol-ether extraction

The procedure adopted here had been used by Tatum and co-workers (1936) in their work on the potato fractions active for *Lactobacillus delbruckii*. Five volumes of 95 per cent alcohol were added to one volume of hydrolysate, then six volumes of ether were added. The mixture was shaken continuously while the ether was added. The precipitate was centrifuged down and taken up in distilled water. The alcohol-ether solution was concentrated, freed of organic solvents, and diluted to volume with distilled water. Only the alcohol-ether precipitate fraction, containing the dark material in the hydrolysate, contained essential growth materials, inasmuch as it supported growth in the absence of added amino acids. The alcohol-ether soluble fraction was negative alone and in the presence of added amino acids (mixture 3).

3. Treatment with lead acetate and ammonia

Although admittedly a remote possibility, it was decided to check the hydrolysates of gelatin and casein for the presence of accessory substances hydrolyzable to glucose. The hydrolysates

were first alkalinized with excess ammonia. A saturated solution of lead acetate was added until no further precipitation occurred. The filtrate was boiled to remove ammonia, saturated with hydrogen sulfide to remove the lead, boiled to remove excess hydrogen sulfide and brought to volume. The lead acetate precipitable fraction was taken up in a little water, saturated with hydrogen sulfide to remove the lead as before and worked up to volume. The growth-essential materials of the hydrolysate were contained in the lead-acetate-soluble fraction which alone supported growth. The insoluble fraction was of no value.

4. Treatment with norite

A portion of the original hydrolysate and also an aliquot of the lead-acetate-soluble fraction were treated with purified animal charcoal, boiled for an hour and filtered twice through Whatman #40 paper. The charcoal precipitates were washed twice with hot water and then dried. The filtrates and washings were combined and concentrated on the water bath to their original volume. Growth tests revealed that the essential materials had been completely removed by the norite.

Boiling the charcoal for a few hours with 95 per cent ethyl alcohol eluted the growth materials with much loss involved. A fraction supplementary only in effect could not be obtained.

5. Effect of the Neuberg-Kerb reagent (1911)

The hydrolysate was treated with sufficient 10 per cent sodium carbonate to alkalinize it. To this, a 25 per cent solution of mercuric acetate was added until a permanent brick-red precipitate appeared. Five volumes of 95 per cent alcohol were added. The mixture was then filtered. The filtrate and precipitate were freed of mercury by hydrogen sulfide and concentrated to volume on the water bath. The growth-essential materials were concentrated in the mercury-insoluble fraction. The soluble fraction was negative in the growth tests.

All growth tests with the above fractions were carried out in two basal media, (1) one containing only monobasic potassium phosphate, sodium hydroxide, tryptophane, sporogenes vitamin

and thioglycollic acid in amounts as on page 433, (2) the other being amino acid mixture lot 3.   The fractions under test were added in amounts equivalent to that of the original protein hydrolysate which supported growth.

The results are summarized in table 5.

The inability to produce a fraction which was only supplementary in action to the sporogenes vitamin (i.e., which acts *only in basal medium number 2*) is indicated by the above table.

It was therefore decided to prepare another lot of sporogenes

TABLE 5

Activity of gelatin hydrolysate fractions

FRACTION	INCUBATION PERIOD		TOXIN
	Basal medium 1	Basal medium 2	
Original hydrolysate..............................	++++	++++	+
Alcohol-soluble fraction............................	+++	+++	+
Alcohol-insoluble fraction..........................	0	0	0
Alcohol-ether soluble fraction......................	0	0	0
Alcohol-ether insoluble fraction....................	+++	+++	+
Lead acetate-ammonia filtrate......................	+++	+++	+
Lead acetate-ammonia precipitate..................	0	0	0
Norite filtrate, pH 7.0............................	0	0	0
Neuberg-Kerb filtrate.............................	0	0	0
Neuberg-Kerb precipitate (0.5 cc.)..................	+	+	0
Neuberg-Kerb precipitate (1.0 cc.)..................	++	++	0
Neuberg-Kerb precipitate (3.0 cc.)..................	+++	+++	+
Ethyl alcohol eluate of Norite-adsorbed material.....	+++	+++	+

vitamin, this time using pregnancy urine as a source of the material and adopting Pappenheimer's published method (1935) for the latter stages of the work.   The preliminary treatment of the urine was carried out in accordance with some suggestions kindly sent to me by Dr. Pappenheimer.

One hundred liters of freshly obtained pregnant cow's urine were concentrated to one-tenth of the original volume by distillation *in vacuo*.   Equal volumes of normal sulfuric acid and butyl alcohol were added to the concentrate and boiled for twenty-four hours under a reflux condenser.   The butyl alcohol layer was

removed and concentrated *in vacuo* to a thick tar. This was partially dissolved in pyridine. An equal volume of peroxide-free ether was added and the insoluble matter removed. The material was then extracted with concentrated acid and the acidified extract alkalinized with sodium carbonate. The rest of the procedure follows Pappenheimer's description (1935).

The yellow material obtained was very active, 1×10^{-7} gram activating 1 ml. of the amino acid basal mixture developed by Fildes for *C. sporogenes*. However, the growth obtained with this amount of vitamin, and with increasing amounts also, did not compare favorably with the growth which the same amount of vitamin supported in gelatin hydrolysate media. One or more essential substances appear to be required for the growth of *C. parabotulinum* in synthetic amino acid mixtures, in addition to the active substance in yeast and pregnancy urines. A further investigation of these findings is being carried on.

SUMMARY

1. Previous reports concerning the essential nature of certain acidic fractions from yeast and pregnancy urines in the growth of *Clostridium parabotulinum* have been confirmed.

2. Growth of *Clostridium parabotulinum* in media composed of amino acids has been obtained.

3. Although the nutritional requirements of *Clostridium parabotulinum* resemble those of *Clostridium sporogenes* in the group of amino acids required for growth, there are qualitative differences in their needs which have not yet been solved to the point of obtaining massive growth in synthetic media.

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THE LETHAL ACTION OF SHORT ULTRAVIOLET RAYS ON SEVERAL COMMON PATHOGENIC BACTERIA

D. G. SHARP

Departments of Surgery and Bacteriology, Duke University School of Medicine and Duke Hospital

INTRODUCTION

The inhibitory and lethal effects of certain of the shorter ultraviolet rays on bacteria and fungi have been known for about fifty years. Recently, interest has been revived in problems concerning bacteria floating in the air especially in relation to the sterilization of air as a protection against air-borne infections. (Wells and Wells, 1936 and 1938, Hart, 1937 and 1938, and Sharp, 1938). Since a quantitative evaluation of the resistance to radiation of most of the bacteria against which ultraviolet-ray protection is needed has not been made, such comparative study seems desirable.

Careful quantitative estimates of the energy lethal for various species of bacteria have been made by Coblentz and Fulton (1924) on *Escherichia coli*, Gates (1929 and 1930) on *Staphylococcus aureus* and *Escherichia coli*, Wyckoff (1931) on *Escherichia coli*, Ehrismann (1931) on *Escherichia coli*, *Staphylococcus aureus*, *Serratia marcescens*, etc., and Hollaender and Claus (1936) on *Escherichia coli*. In the last paper, the results of the previous work are summarized. Through a comparison of the data obtained by different experimental methods, attempts are made to reduce all the results to the common denominator of lethal energy per bacterium. It is notable that, while all the studies cited include some of the same species of bacteria, the absolute lethal energies given for a particular wave length differ substantially.

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Studies of spore-forming bacteria (*Bacillus subtilis* by Duggar and Hollaender, 1934 and *Bacillus megatherium* by Hercik, 1936) indicate that approximately twice as much energy is necessary to kill the spores as is needed for the vegetative forms.

In none of the studies in which absolute energy values have been measured has more than slight attention been given to the common pathogenic bacteria. The work of Dreyer and Campbell-Renton (1936) gives lethal curves for several pathogens but the light intensities were measured in arbitrary units and are therefore not directly comparable with other quantitative work.

The experiments reported here were concerned with the determination of the relative resistance of several pathogens, notably the more important pathogenic air contaminants, as well as *Staphylococcus albus*, *Escherichia coli*, and *Serratia marcescens*, to the unfiltered rays of a low-pressure mercury glow lamp[1] of the type used for sterilization of air in operating rooms. Its characteristics have been described elsewhere (Sharp, 1938) and will be reviewed only briefly here.

<div align="center">METHOD</div>

The highly pathogenic nature of several of the species of bacteria studied makes measurement in air suspension dangerous. Previous work (Sharp, 1938) has shown that in the instance of *S. albus* the amount of energy necessary to kill 100 per cent in air suspension (presumably saturated air) is of the same order of magnitude as that given by the plate method. It was considered advisable to employ the latter method.

Standard nine-centimeter Petri plates containing solid media suitable for the growth of the organism under observation were seeded uniformly with bacteria in the following manner: A twenty-four-hour broth culture was diluted with normal saline solution until it contained about fifteen thousand organisms per cubic centimeter. Five cubic centimeters of this were pipetted on to the agar surface of each plate and kept in motion for twenty

<hr>

[1] This lamp is manufactured by Westinghouse Electric and Manufacturing C. under the name of "Sterilamp."

seconds; the plate was then inverted for about forty seconds to drain off the excess liquid. By this means 600 to 1,000 evenly distributed colonies could be obtained on each plate (fig. 1).

Prepared plates were exposed to ultraviolet rays in a frame (fig. 2) at a distance of 35 inches from a pair of radiation tubes and
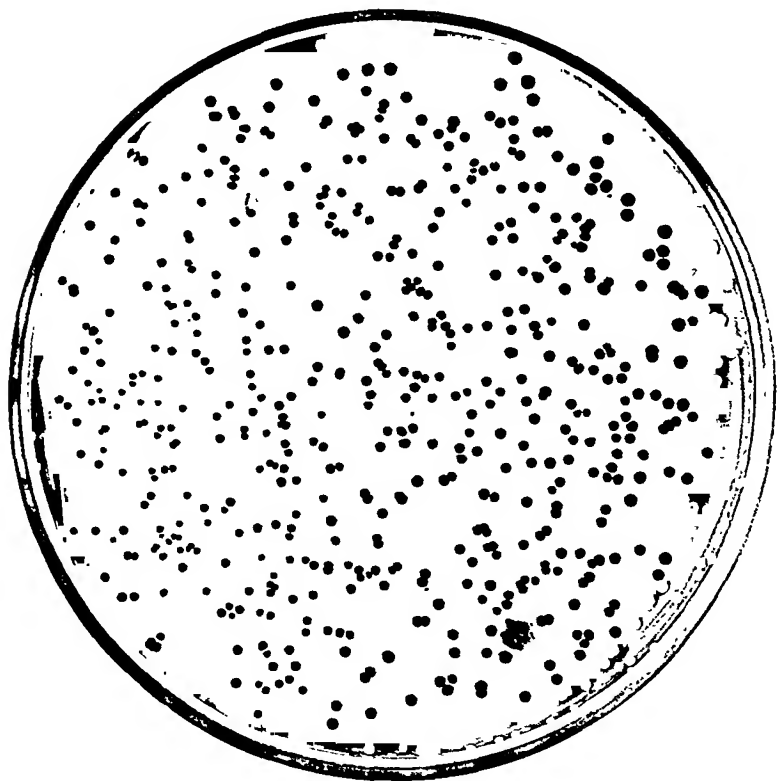
FIG. 1

in a plane normal to the rays. The tubes were 30 inches long, operated at about five degrees above room temperature and consumed 5 to 10 watts power each. They are particularly well adapted to this work since over 85 per cent of the total radiant output is in the resonance 2537 Å line. There are no other lines in the region below 3000 Å strong enough to have appreciable

bactericidal effect so that it is safe to assume, for present purposes, that the unfiltered rays are monochromatic and as such, comparable in their effect with those usually isolated with a monochrometer. This allows a direct comparison of results obtained here with those of other observers.

Immediately below the plates (fig. 2) and in a similar position with respect to the lamps was mounted a special ultraviolet dosimeter (Rentschler, 1930) consisting of a tantalum-target photoelectric cell and relay apparatus suitable for metering the energy received. This device recorded the actual amount of
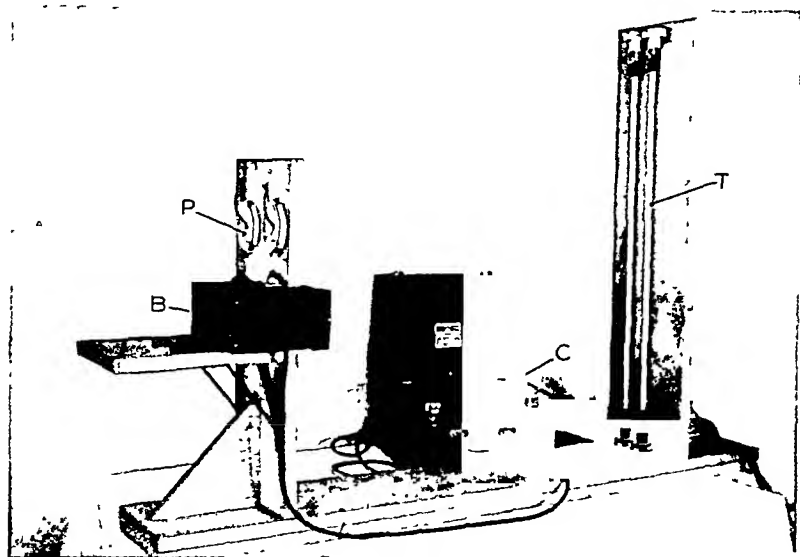
FIG. 2

energy received over a given length of time and so was free from errors that might have been caused by variation in lamp intensity. Standardization was effected by comparison with a bismuth-silver vacuum thermopile and National Bureau of Standards radiation constant. All energy values are given directly in incident ergs per square millimeter of exposed surface.

RESULTS

For each organism used, two separate groups of 12 to 15 plates were exposed on different days. Exposures were selected to give

the best possible determination of the amount of energy necessary to reduce the number of surviving bacteria to 10 per cent of the original number.   No attempt has been made to examine closely the extremes of the reaction, namely; near 0 per cent and near 100

TABLE 1

ORGANISM	MEDIUM (pH 7.4)	INCIDENT ENERGY ERGS/ MM² FOR 90 PER CENT KILLING (10 PER CENT SURVIVAL)	OTHER OBSERVATIONS
Bacillus anthracis (mixed spores and veg. forms).....	Beef-extract agar	452	
Corynebacterium diphtheriae...	Beef-infusion blood agar	337	
Staphylococcus aureus-hemolyticus..................	Beef-extract agar	260	218 ergs, Gates (1929) 600 ergs, *Ehrismann and Noethling (1931)
Escherichia coli..............	Beef-extract agar	245	150 ergs, *Ehrismann and Noethling (1931) 640 ergs, Wyckoff (1931)
Serratia marcescens...........	Beef-extract agar	220	82 ergs, *Ehrismann and Noethling (1931)
Streptococcus hemolyticus.....	Beef-extract blood agar	216	
Eberthella typhosa............	Beef-extract agar	214	
Streptococcus viridans........	Beef-extract blood agar	200	
Staphylococcus albus..........	Beef-extract agar	184	
Shigella paradysenteriae......	Beef-extract agar	168	

Table showing incident energy at 2537 Å necessary to reduce several species of bacteria to a survival ratio of 10 per cent (90 per cent killed).
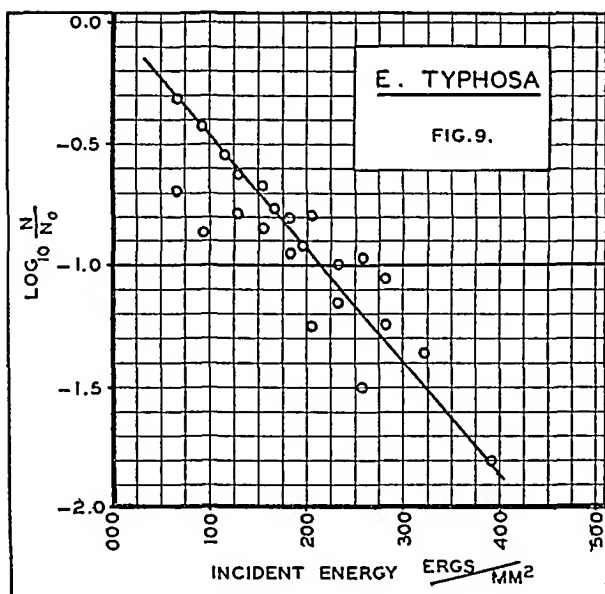*Values based on 90 to 100 per cent killing.

per cent survival.   This has been done by others, (Hollaender and Claus, 1936) for non-pathogenic bacteria, and it was not considered advisable to repeat it here since the exposure of many more plates would have been necessary.
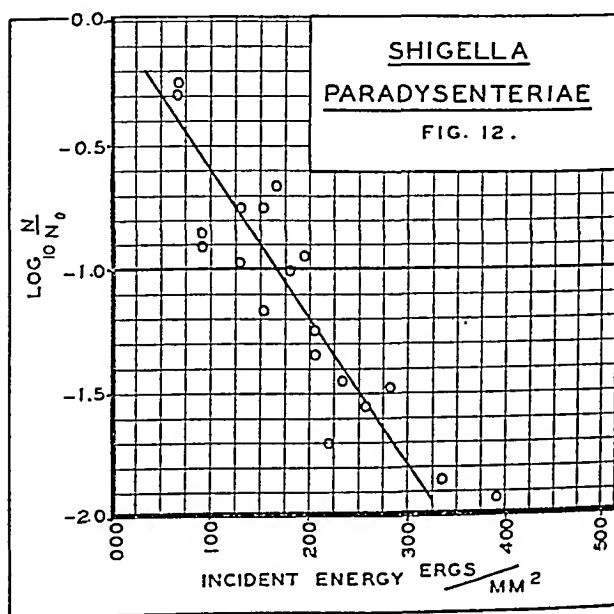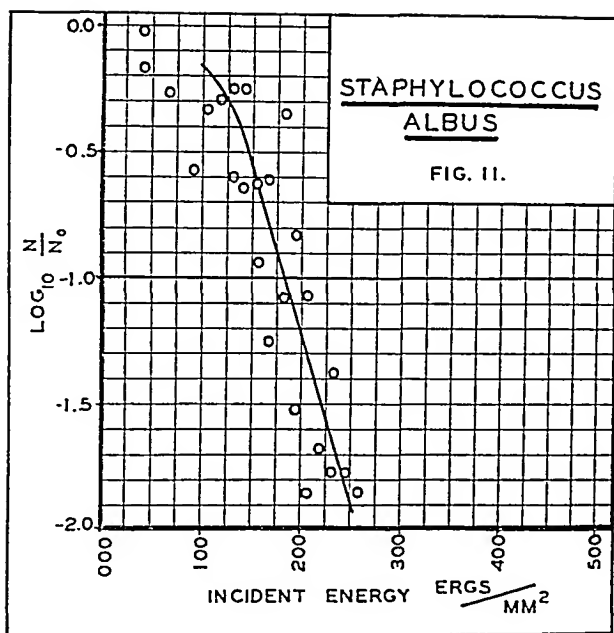
Figures 3 to 12 show the logarithm of the survival ratio plotted against the incident energy in ergs per square millimeter and the

FIG. 3.

B. ANTHRACIS

SPORES & VEG. FORMS

FIG. 4.

C. DIPHTHERIAE

HEMOLYTIC
STAPHYLOCOCCUS
AUREUS

FIG. 5.

E. COLI

FIG. 6.

S. MARCESCENS (PRODIGIOSUS)

FIG. 7.

STREPTOCOCCUS HEMOLYTICUS

FIG. 8.

E. TYPHOSA

FIG. 9.

STREPTOCOCCUS

VIRIDANS

FIG. 10.

STAPHYLOCOCCUS ALBUS

FIG. 11.

INCIDENT ENERGY ERGS/MM²

SHIGELLA PARADYSENTERIAE

FIG. 12.

INCIDENT ENERGY ERGS/MM²
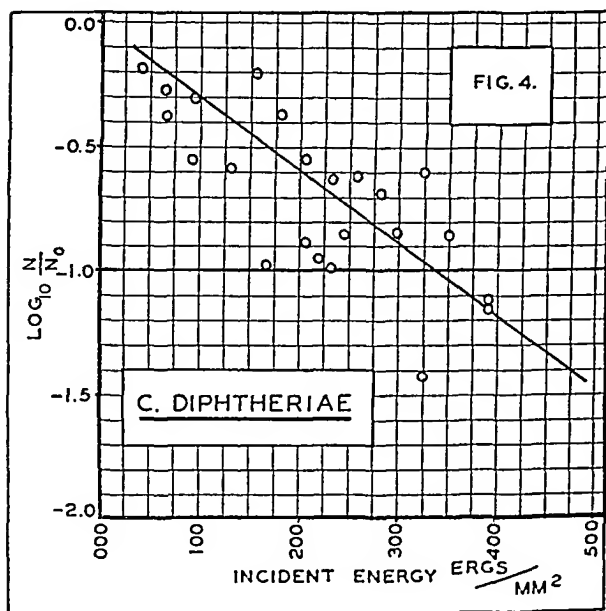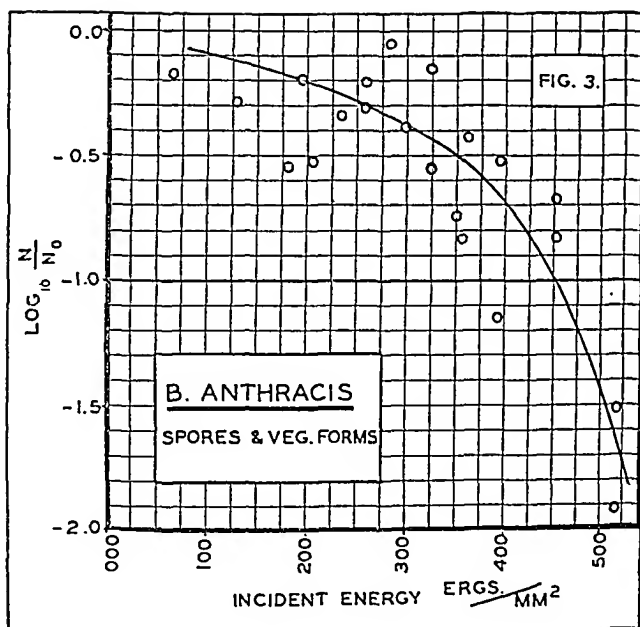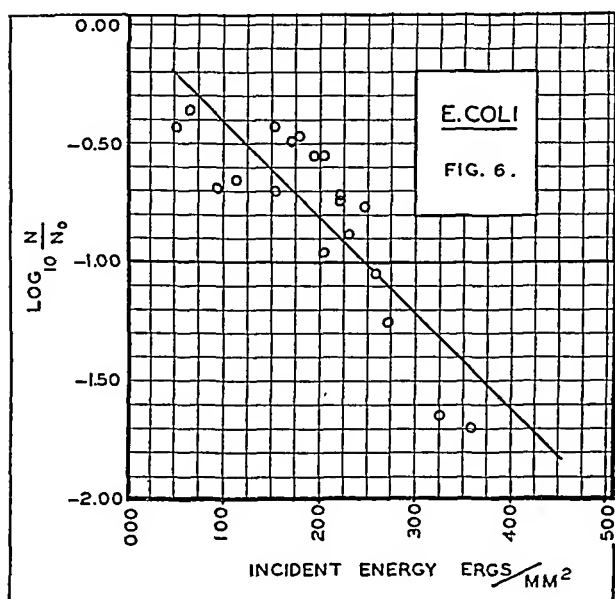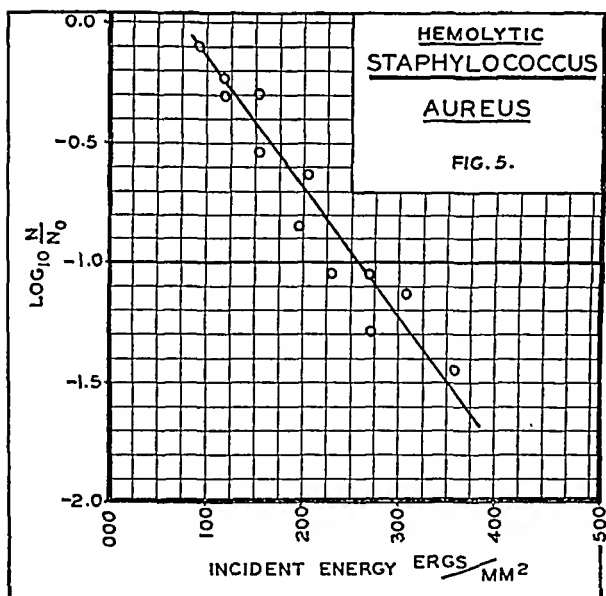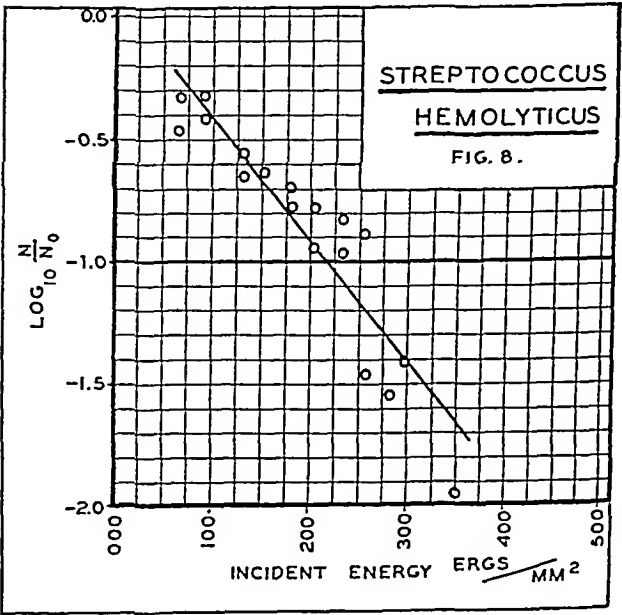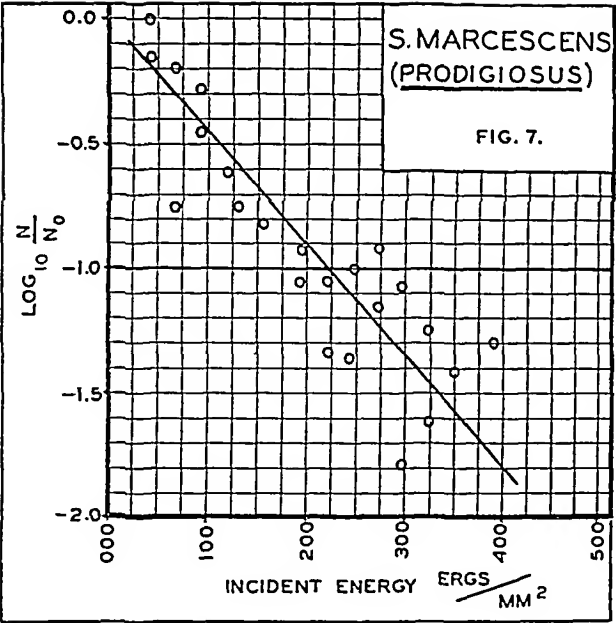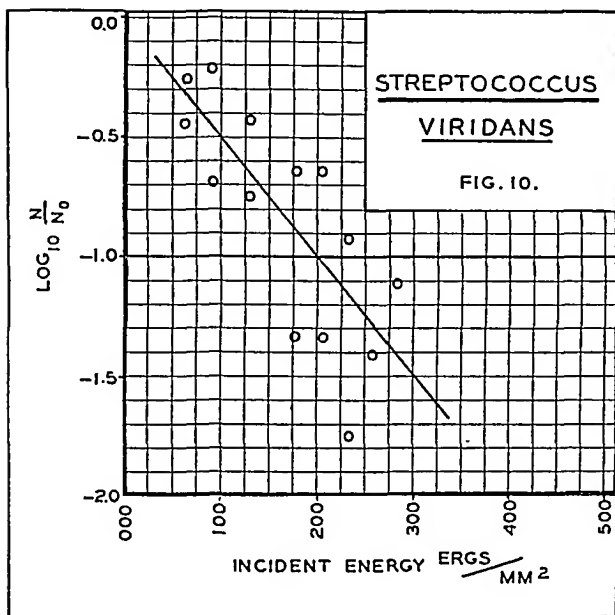
curves drawn through the points. The intersections of these curves with the ordinate line -1 (10 per cent survival) is the basis on which the bacteria are compared (table 1). The intensity was constant and such that the time required to give this amount of energy was about 2 minutes.

DISCUSSION

It has been shown for several types of bacteria, (Wyckoff, 1931, Hollaender and Claus, 1936, and Hercik, 1936), that the number dying per unit of incident energy is approximately in direct proportion to the number remaining alive in the culture, i.e.:

$$\frac{dN}{dE} = -k_1 N$$

E = energy incident on culture,
N = number of bacteria remaining ·after a given amount of energy has been applied.

$$\log \frac{N}{N_0} = -k_2 E$$

This, when integrated from energy $E = O$ to any given amount E, gives the corresponding survival ratio $\frac{N}{No}$.

No = number of bacteria originally on the plate as given by the controls.

The constant K_2 depends on the absorption coefficient of the particular species and on the wavelength of the light. Although certain deviations from this straight-line relationship between $\mathrm{Log}\,\frac{N}{No}$ and the energy have been reported, it serves as a convenient method of comparison of species resistance and is used here to facilitate comparison with other data.

A direct comparison can be made in the case of *Staphylococcus aureus* with the data of Gates (1929) which show 218 ergs/mm² necessary to kill 90 per cent of these organisms on agar surface. From our data (fig. 5) it is seen that 260 ergs/mm² were necessary

for our strain of *S. aureus.* The same author (Gates, 1930) found 110 ergs/mm² necessary to reach 50 per cent killing of *E. coli* and our data (fig. 6) would indicate 75 ergs/mm² for this organism. Wyckoff (1931), on the other hand, found 640 ergs/mm² necessary for 90 per cent killing while curve 6 shows only 245 ergs/mm². Only approximate comparisons with the studies of Ehrismann and Noethling (1931) are possible since their estimates were made on the broad basis of 90 to 100 per cent killing. Their figures have been entered in table 1, and it will be seen that large variations occur. No such wide differences in resistance have been found among the various species tested here. In fact, all the figures for 90 per cent killing of non-sporing organisms fall between 168 ergs/mm² (*Shigella paradysenteriae*) and 337 ergs/mm² (*Corynebacterium diphtheriae*).

Bacillus anthracis, which showed more resistance than any of the nonsporing species, was killed by about twice the energy necessary to kill *E. coli*. This was true even if complete sterilization instead of 90 per cent killing was considered as the point of comparison. The results of Hercik (1937) on *Bacillus megatherium* indicate, too, about twice as much energy required to kill spores as was required for vegetative forms. The curve (fig. 3) seems to indicate by its increasing rate of fall that there is an apparent decrease in resistance rather than the expected increase due to surviving spores.

The results summarized in table 1 indicate that the resistance to 2537 Å rays of the several species of bacteria was not only of the same order of magnitude but that the most resistant non-spore producer (*C. diphtheriae*) was only about twice as resistant as the least resistant (*S. paradysenteriae*).

It is to be expected that the ultraviolet ray resistance of these bacteria in air suspension would be subject to certain variables not present in this experiment. Wells (1936) has reported wide differences in bacterial resistance with changes in the relative humidity of the air. His findings indicate a reduced resistance in air of lower humidity. In the light of his observation our previous work on *S. albus* done in air (Sharp 1938) under conditions near saturation could be taken to indicate the maximum

resistance of the organism. The present data taken from bacteria on moist plate surfaces might be subject to the same considerations. The lethal energies in both experiments were found to be of the same order of magnitude.

SUMMARY

The following species of bacteria were tested for resistance to short ultraviolet rays, and the energy necessary to reduce each to a 10 per cent survival ratio is recorded: *Eberthella typhosa, Shigella paradysenteriae, Corynebacterium diphtheriae, Staphylococcus aureus (Hem), Staphylococcus albus, Streptococcus viridans, Streptococcus hemolyticus, Bacillus anthracis* (mixture of spores and vegetative forms), *Escherichia coli* and *Serratia marcescens*.

The mixture of the spore and vegetative forms of *Bacillus anthracis* was not more than twice as resistant as *Escherichia coli*. This was true even when 100 per cent killing or zero survival was considered as a reference point.

Unfiltered radiation (85 per cent 2537 Å) from a commercial low pressure glass mercury arc was used. Its degree of purity was sufficiently great to make a monochrometer unnecessary for bacterial work, and regular 9 cm. Petri plates can be conveniently exposed to a uniform field of rays.

A commercial ultraviolet ray dosimeter, calibrated in absolute units, was used to measure the energy. The sensitive agent is a tantalum-target vacuum photoelectric cell.

It was found that the extreme values of energy necessary to kill 90 per cent of non-sporing organisms were 168 ergs/mm^2 (*Shigella paradysenteriae*) and 337 ergs/mm^2 (*Corynebacterium diphtheriae*). For a mixture of spores and vegetative forms of *Bacillus anthracis*, however, 452 ergs/mm^2 were necessary. The results obtained are compared with the data of other observers.

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PRECIPITINS FOR THE TUBERCULIN PROTEINS OF ACID-FAST BACTERIA[1]

JANET McCARTER, ELIZABETH M. KANNE, AND E. G. HASTINGS

Department of Agricultural Bacteriology, University of Wisconsin

INTRODUCTION

Seibert (1930) found that the tuberculin proteins of the three varieties of tubercle bacillus could be distinguished by the precipitin test. If this method could be adapted for identifying tubercle bacilli, it would be preferable to animal inoculation. The behavior of the tuberculin proteins[2] of acid-fast bacteria in the precipitin test is interesting not only as a possible means of identification of the bacilli, but also because the proteins and their derivatives are apparently the active agents in tuberculins.

METHODS

The cultures investigated included one strain of the human tubercle bacillus; one strain of the bovine; five strains of the avian, three isolated from chickens, one from a hog, and one from a cow; one strain of Johne's bacillus; and two cultures of so-called saprophytic acid-fast bacteria, one isolated from a cow and one from a hog.[3] After the growth on the synthetic medium of

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[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation and is published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[2] By "tuberculin proteins" we mean proteins in culture filtrates as distinguished from proteins isolated directly from cells.

[3] The human strain was obtained from the Bureau of Animal Industry and has been used in our laboratory for making Old Tuberculin for twenty years. The bovine strain, isolated by Traum, was obtained from the Cornell Veterinary College. The avian strains, the Johne's bacillus, and the two saprophytic strains were all isolated in this laboratory. The Johne's bacillus grows without phlei cells. See discussion for description of saprophytic strains.

Dorset and Henley (1934) had autolyzed, usually three months after inoculation, the cells were removed by a paper and then by a Mandler filter. To the filtrate was added an equal volume of one-per-cent phenol. It was then concentrated to about one-tenth of the original volume by ultrafiltration and washed with 0.5 per cent phenol until a BaCl$_2$ test for sulphate was negative. Concentration was continued until the solution contained from 0.5 to 1.0 mgm. of protein per cubic centimeter. It was then filtered through a Berkefeld filter and handled aseptically thereafter. The amount of protein in the solution was estimated according to Seibert's (1928) method: precipitation of a 5-cc. sample with 5 cc. of 20-per-cent trichloracetic acid, centrifugation until a constant volume of precipitate was obtained, and computation for protein content from Seibert's determination on a human tubercle bacillus protein that 1 cc. of precipitate contains 0.0674 gram of protein. Nitrogen determinations on some of the protein solutions, by a modification of the micro-Kjeldahl method involving Nesslerization and colorimeter readings, showed from 10.5 to 12.5 per cent more protein than was estimated by Seibert's standardization method. As a precaution against one protein contaminating another, the Mandler and Berkefeld filters were cleaned after each use by soaking over night in a hot 0.5-per-cent sodium hydroxide solution; all glassware was cleaned with chromic acid cleaning solution, and new ultrafilters were used for each protein.

The precipitins were incited in rabbits by the subcutaneous injection of the antigens in solution, 3 to 5 cc. being given at intervals of two to three days until a total of about 25 mgm. of protein had been injected. In the first part of the work the rabbits were bled three weeks after the last injection, but later it was found that the sera taken one week after the last injection showed more antibody.

In making the precipitin tests, 0.1 cc. of the antigen solution was layered with a capillary pipette on 0.1 cc. of the antiserum. The antigen was tested in strengths starting from one gram of protein in 1250 or 2500 cc., each successive dilution being twice that of the preceding. Dilutions were usually made with 0.2

per cent salt solution, although concentrations of from 0.2 to 0.9 per cent gave no apparent differences in the results.. Readings for rings were made after two hours at 37°C. and for precipitate after an additional 12 or more hours in the icebox. A lighting device described by Kanne and McCarter (1939) was used in examining for rings and precipitate.

In carrying out the precipitin absorption tests, preliminary tests were made with a constant volume of serum and varying quantities of antigen to determine the ratio necessary for the maximum amount of precipitate. In making the absorptions, apparently as much precipitate was formed if incubation was carried out wholly at 0° as if carried out partially at 37°.

<div align="center">RESULTS</div>

The results of the precipitin tests with undiluted antisera and the tuberculin proteins of the various acid-fast bacteria are given in table 1. The tests with the human and bovine antisera are not conclusive, since the proteins of only one strain of each of the three varieties of tubercle bacilli were tried. However, these results agree with Seibert's (1930) in that the proteins of the three varieties of tubercle bacilli and of the two saprophytes were all distinct. The avian protein, even though it reacted in nearly as high a dilution as the human protein with the human antiserum, could be distinguished easily because it gave much less precipitate in corresponding dilutions. With the avian and Johne's antisera, the avian tubercle bacilli from the different sources and the Johne's bacillus could not be distinguished from each other, but could be distinguished from the human and the bovine types. Although the bovine protein reacted in high dilutions with the avian and Johne's antisera, in corresponding dilutions much less precipitate was obtained with the bovine protein than with any of the avian proteins or the Johne's protein. Apparently comparable amounts of precipitate with Johne's and avian proteins were obtained in both Johne's and avian antisera.

Tables 2 and 3 show attempts to differentiate the Johne's and avian proteins by precipitin absorptions and by antiserum

TABLE 1
Precipitin tests with tuberculin proteins and their antisera

ANTISERUM (UNDILUTED)	ANTIGEN TITER*									
	Human t.b.	Bovine t.b.	Avian t.b. 1 (chicken)	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Avian t.b. 5 (hog)	Johne's bacillus	Saprophytic acid-fast (cow)	Saprophytic acid-fast (hog)
Human .	10,000		5,000						0	0
Bovine	10,000	40,000	5,000						2,500	0
Avian 1 .	0		20,000						0	0
Avian 2	10,000	80,000	160,000	320,000	80,000	40,000	160,000	320,000	20,000	10,000
Avian 4 .	0	40,000	80,000	80,000	40,000	80,000	80,000	80,000	10,000	
Johne's	10,000	80,000		160,000	160,000	80,000	160,000	160,000		5,000
Saprophyte (cow) ..	0	0	5,000						40,000	0

* The titer is given as the highest dilution (expressed as cubic centimeters of solution containing 1 gram of protein) of the protein solution showing a ring or precipitate as compared with a control of antiserum and salt solution. The titers for rings and precipitate were usually the same, although, when the lighting device was used, definite rings could sometimes be seen with higher dilutions than showed precipitates.

TABLE 2
Precipitin absorptions

TUBERCULIN PROTEIN WITH WHICH ANTISERUM ABSORBED	ANTIGEN TITER					
	Human t.b.	Bovine t.b.	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Johne's bacillus
Antiserum for the tuberculin protein of avian t.b. 4 (diluted 1 to 4)						
Salt solution control.... .	0	640,000	320,000	320,000	640,000	640,000
Avian t.b. 4		0	0	0	0	0
Avian t.b. 3		0	0	0	0	0
Bovine t.b.......		0	10,000	80,000	160,000	80,000
Antiserum for the tuberculin protein of the Johne's bacillus (diluted 1 to 4)						
Salt solution control.......	5,000	160,000	80,000	160,000	80,000	320,000
Avian t.b. 4....	0	0	0	0	0	0
Avian t.b. 3	0	0	0	0	0	0
Bovine t.b		0	20,000	80,000	80,000	160,000

TABLE 3
Precipitin tests with diluted antisera

ANTISERUM (DILUTED 1 TO 32)	ANTIGEN TITER					
	Bovine t.b.	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Avian t.b. 5 (hog)	Johne's bacillus
Avian 4...	0	40,000	80,000	320,000	0	80,000
Johne's..............	0	0	40,000		160,000	160,000

dilution. In the precipitin absorption tests, absorption of both avian and Johne's antiserum with bovine protein removed only bovine-reacting precipitins, while absorption with avian protein removed bovine, avian, and Johne's precipitins. The Johne's protein never completely absorbed precipitins for either the avian or Johne's proteins and is, therefore, not included in the table. When the antisera were diluted 1 to 32, as in table 3, the bovine-protein-reacting precipitins were eliminated but the Johne's and avian were still not distinguishable. Further dilution eliminated both Johne's and avian precipitins.

<div align="center">DISCUSSION</div>

That the proteins are the active antigens in tuberculins has been assumed in the interpretation of our results. This assumption is made on the basis of the finding by Seibert and Munday (1931) that the antigenicity of tuberculins in the precipitin test is correlated with high nitrogen content and large molecular size rather than with carbohydrate content, although polysaccharide is combined in some way with the protein in ultrafiltered tuberculins. This polysaccharide is removed from the protein derivatives in the P.P.D. (Purified Protein Derivative) tuberculin by repeated precipitation of the protein solution with trichloracetic acid. Since we wished to alter the protein as little as possible, and were interested in finding a test for identifying acid-fasts which would involve as few manipulations as possible, we did not remove the carbohydrate from the protein solution.

On the basis of our results, human, bovine, and avian tuberculin proteins could be distinguished from each other by the precipitin test. The separation of human from avian protein is evident from table 1, since the avian always gave much higher titers with avian and Johne's antisera. The bovine was separated from the avian on the basis of a much higher titer with bovine antiserum and much less precipitate with avian or Johne's antiserum in comparable dilutions. Also, the bovine protein did not absorb all the avian or Johne's precipitins from either the avian or the Johne's antiserum but only reduced them slightly. (We did not test the capacity of the bovine protein to absorb com-

pletely the bovine antiserum, and therefore would not draw conclusions from the absorption test alone, since the antigen in question has not been shown to absorb completely its own or a heterologous antiserum.)

The proteins of the three avian strains from chickens, the strain from the cow, and the strain from the hog, all behaved so nearly alike in the precipitin test as to be indistinguishable. Some variations do occur in the behavior of the different avian proteins in the antisera for the avian tubercle bacillus strains 2 and 4, and in the diluted antiserum for strain 4. These variations can be attributed to varying amounts of different proteins in the same tuberculin or of different reactive groups on any one protein. Seibert, Pedersen, and Tiselius (1938) have found that even the P.P.D. tuberculin contains several proteins or protein derivatives of different molecular weights. The bovine protein apparently reacts in avian antisera with the antibody for only part of the proteins or reactive groups.

The surprising result was that the Johne's bacillus protein could not be distinguished from the avian tubercle bacillus proteins in either undiluted or diluted antisera. The precipitin absorption tests gave corroborative evidence that the tuberculin proteins of the Johne's bacillus and of avian tubercle bacilli are closely similar, since the proteins of the avian strains 3 and 4 removed all precipitins from the antisera for the Johne's protein and for the avian (strain 4) protein.

The Johne's bacillus and the avian tubercle bacillus have widely different cultural characteristics and pathogenic capacities. Evidently, however, they have a common protein and therefore cannot be identified by the precipitin test. Consequently the test can not be used by itself to identify an unknown acid-fast, but can be used to give evidence supplementary to the cultural and pathogenic characteristics. Thus, the culture of avian tubercle bacilli from the cow, designated as strain 4, reacts as an avian or Johne's strain in the precipitin tests; has the cultural characteristics of the avian tubercle bacillus; is pathogenic for rabbits and not for guinea pigs; but does not produce pro-

gressive tuberculosis in chickens (a full description of this culture will be published).

The similarity of the tuberculin proteins of the Johne's bacillus and the avian tubercle bacillus is interesting because it gives a stronger basis for the use of avian tuberculin in the diagnosis of Johne's disease, which Hagan and Zeissig (1927–28) have advocated on the basis of clinical findings. It had been thought that this might be because both avian tubercle bacilli and Johne's bacilli are so often isolated from animals having Johne's disease. On the basis of the precipitin test, the common proteins probably account for the success of the diagnostic test.

That neither of the two saprophytes tested is a variety of tubercle bacillus was established by the precipitin test. The one microorganism was isolated from a no-visible-lesion tuberculin-reacting cow. Injection of this culture sensitized another cow to human tuberculin, and Feldman (1933) found that it sensitizes chickens to avian tuberculin. The other saprophyte was isolated from a hog, from lymph nodes showing caseous pinhead-sized tubercles. Neither culture is pathogenic for rabbits, chickens, or guinea pigs, and both cultures have quite similar cultural characteristics. The protein of the saprophyte from the hog does not give any cross reaction in the antiserum for the protein of the saprophyte from the cow; indicating that the two saprophytes are different species.

The occurrence of cross-reactions with the avian tuberculin protein in the antisera for the mammalian (human and bovine) tuberculin proteins, and with the mammalian proteins in the avian antisera, is in agreement with the findings that all human beings sensitive to mammalian tuberculin react to avian tuberculin (McCarter, Getz, and Stiehm (1938)), and that cattle infected with avian tubercle bacilli react to mammalian tuberculin (McCarter, Beach and Hastings (1937)).

Since the tuberculin proteins of the three varieties of tubercle bacilli, of the Johne's bacillus, and of the two saprophytes all reacted in more than one heterologous antiserum, it is doubtful whether a tuberculin test with any of the available tuberculins

can be used to diagnose for infection with a specific acid-fast microorganism; i.e., whether a reaction to a specific tuberculin necessarily means that the animal tested has been infected with the acid-fast from which the tuberculin was made.

CONCLUSIONS

1. The tuberculin proteins of the human, the bovine, and the avian tubercle bacillus are distinguishable by the precipitin test.

2. The tuberculin proteins of the avian tubercle bacilli isolated from the chicken, the cow, and the hog, and the protein of the Johne's bacillus can not be differentiated by the precipitin test.

3. The tuberculin proteins of two so-called saprophytic acid-fast bacteria, one isolated from the cow and one from the hog, are distinguishable from each other and from the proteins of the tubercle bacilli and of the Johne's bacillus.

4. The precipitin test with tuberculin proteins as antigens is useful in identifying unknown acid-fast bacteria when considered in conjunction with cultural and pathogenic characteristics. Further purification of the protein solutions seems necessary before the precipitin test can replace other methods of identification of acid-fasts.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

WASHINGTON BRANCH

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., FEBRUARY 21, 1939

OXIDATION OF PERSEITOL BY ACETO-BACTER SUBOXYDANS. *E. B. Tilden,* Division of Chemistry, National Institute of Health, Washington, D. C.

Perseitol (α-d-mannoheptitol), obtained from the seeds of the avocado, was oxidized by Bertrand, in 1908, to a crystalline ketoheptose (perseulose) by using the alcohol as carbon source in yeast extract cultures of his "sorbose bacterium" (*Acetobacter xylinum*). Bertrand obtained 45% yields of the sugar. The preparation of perseulose in quantity for chemical studies was the object of the present work.

In the bacterial method of preparation of sorbose, Kluyver and de Leeuw's *Acetobacter suboxydans* had recently been found to give better yields than *A. xylinum*. It also grows in a homogeneous suspension in culture. While the bacteria oxidize a commercial sorbitol syrup nearly quantitatively in the presence of 0.5% yeast extract alone, only 30% perseitol was oxidized under the same conditions. However, complete oxidation was obtained by addition to the yeast extract of a small amount of glucose (0.05%), together with phosphate buffer. The optimum pH for the reaction was 6.0–6.8. The glucose-phosphate-yeast extract appears to be a suitable medium for the oxidation of other pure sugar alcohols by *A. suboxydans,* as judged from preliminary tests in which the presence of reducing sugar was determined by Shaffer Hartmann tests.

EFFECT ON FACTORY CANE JUICES AND SIRUPS OF LEUCONOSTOC MESEN-TEROIDES ISOLATED FROM FROST DAMAGED LOUISIANA SUGARCANE OF THE 1937 CROP. *M. A. McCalip,* Carbohydrate Research Division, and *H. H. Hall,* Food Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture.

The gum-producing microörganism, *Leuconostoc mesenteroides,* was isolated from the 1937 crop of Louisiana frozen sugarcane. The gum dextran, produced by the microörganism at the expense of sucrose, greatly increases the viscosity of juice and sirup from the cane, retards filtration and crystallization and introduces difficulty and errors in sucrose determinations. Growth of a pure culture in nonbuffered 10 per cent sucrose solution at 20°C. results in a loss of sucrose, rapid increase in acidity and increase in reducing sugars in which glucose predominates. When the microörganism was cultured in a sugar solution buffered to pH 7.5, the viscosity was increased several fold and crude gum was formed to the extent of approximately 50 per cent of the original

sucrose content. d-Mannitol was recovered from the solutions which underwent viscous fermentation.

A BASIDIOMYCETE CULTURE FROM SPUTUM. *C. W. Emmons*, Division of Infectious Diseases, National Institute of Health, Washington, D. C.

NOTES ON RECENT BOOKS OF INTEREST TO BACTERIOLOGISTS. *M. C. Leikind*, Library of Congress.

ON THE "ACTIVATION" OF THE LACTASE OF ESCHERICHIA COLI-MUTABILE[1]

CHARLES J. DEERE

Department of Chemistry, University of Tennessee School of Biological Sciences, Memphis

The studies reported in this paper were directed to an explanation of the puzzling phenomenon that the non-lactose-fermenting *Escherichia coli-mutabile* contains lactase (Deere, Dulaney and Michelson, 1939). Earlier experiments led us to believe that the antiseptics employed "activated" the lactase which was present, but inactive, in living growing cultures of the non-lactose-fermenting (white) form. Use of an antiseptic was at first necessary in determinations of lactase activity since it protected the digest against contaminants and rendered the preparations incapable of attacking glucose and galactose. It was later found that lactase activity of preparations of the white form did not depend on the presence of, or preliminary treatment with, an antiseptic. Drying of cell suspensions by vacuum distillation suffices to "activate" the lactase.

We have investigated more extensively the effect of drying on the various enzyme activities of these organisms. The drying process, unlike the various lactase "activating" antiseptics which we have employed, only partially inhibits the enzyme system or systems concerned in the metabolism of glucose and galactose. Hence, reduction methods for sugars present great difficulties in evaluating results obtained on digests in which the reducing value is being increased by one process (lactose hydrolysis) and

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[1] The data in this paper are taken from a dissertation presented by Charles J. Deere to the Committee on Graduate Study in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Tennessee, September, 1937.

decreased by another (monose metabolism). Since a portion of the sugar metabolism of *Escherichia coli-mutabile* involves the consumption of oxygen, this oxidative phase apparently being limited to monose metabolism, we have attempted herein to analyze enzyme activities from measurements of oxygen consumption. For brevity we shall designate the enzyme system responsible for oxygen consumption as the "oxidase system." The over-all increase in oxygen consumption by dried organisms in lactose, as contrasted with non-dried organisms in lactose, is to be attributed to a primary hydrolysis of the lactose in the first instance and an only partially destructive effect of the drying process upon the subsequent monose metabolism.

METHODS

Oxygen consumption

Oxygen consumption of cell suspensions was measured in a Warburg apparatus at 37.5°C. Carbon dioxide was absorbed by a roll of filter paper moistened with 0.1 cc. of 20 per cent potassium hydroxide.

Cell preparations

The cells were grown on plain or sugar agar and cell suspensions were prepared as described previously (Deere, Dulancy and Michelson, 1939). One-cubic centimeter quantities of cell suspensions were dried in weighed Warburg flasks by vacuum distillation. The weight of dried cells was determined and 1 cc. of distilled water added to replace that lost in drying. One-cubic centimeter portions of cell suspensions which had not been dried served as controls.

Procedure

To the aqueous suspensions of dried or non-dried cells in the Warburg flasks were added 1 cc. of 1.5 per cent sodium chloride in 0.3 M phosphate buffer (pH 7.0) and 1 cc. of 1.5 per cent sugar solution or (for control) 1 cc. of distilled water. The flasks were attached to manometers and placed in the bath. Fifteen minutes later the vessels were shut off from the air and the pressure

changes recorded at 15-minute intervals.   With samples whose oxygen consumption was high, the vessels had to be opened at each 15-minute interval.   When this was necessary, 1 minute elapsed between successive intervals.

<div align="center">RESULTS</div>

In experiment 1 (table 1) is reported the oxygen consumption of suspensions of 24-hour plain-agar cultures of Garrett white.   In lactose, the dried sample (sample 4) consumed 2.5 times as much oxygen as the non-dried sample (sample 10).   The oxygen consumption of dried cells (sample 8) in glucose was about two-thirds that of non-dried cells in glucose (sample 18).   These results demonstrate a partial inhibition of the oxidase system by drying but an "activating" effect on lactase.   The oxygen consumption of dried cells suspended in glucose is about 3 times that of similarly treated cells suspended in lactose.   This finding is not surprising in view of our previous demonstration (Deere, Dulaney and Michelson, 1939) that cells grown in the absence of lactose have only slight lactase activity.

A similar experiment (experiment 2, table 1) was performed using suspensions of Garrett white which had been grown for 24 hours on 1 per cent lactose agar.   The results are qualitatively no different from the results reported in experiment 1.   Dried cells (sample 8) consumed slightly less oxygen in glucose than non-dried cells (sample 18) but, in lactose, dried cells (sample 4) consumed 3.7 times as much oxygen as non-dried cells (sample 10). Non-dried cells (sample 3) consumed about one-half as much oxygen in the absence of sugar as similarly treated cells suspended in lactose (sample 10).   These results demonstrate again the "activating" effect of drying on the lactase of this organism. Comparison of these results with experiment 1 demonstrates the large increase in lactase content which occurs when this organism is grown on lactose.

We then turned our attention to the behavior of the lactose-fermenting (red) strain in similar experiments.   Experiment 3 (table 1) gives the results obtained with a suspension of a 24-hour growth of Garrett red on 1 per cent lactose agar.   It will be noted

TABLE 1

Oxygen consumption of suspensions of Escherichia coli-mutabile

Experiment Number	1					2					3			4		5			6		
Strain used	White					White					Red			Red		White			Red		
Culture medium	Plain					Lactose					Lactose			Plain		Glucose			Plain		
Sample number	10	4	18	8	3	10	4	18	8	18	4	8	10	16	18	7	16	18	10	16	7
Treatment of cells	Non-dried	Dried	Non-dried	Dried	Non-dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Non-dried	Non-dried	Dried	Dried
Weight of sample, mgm.	—	0.3	—	0.2	—	—	0.1	—	8.9	—	0.1	0.0	—	10.0	—	7.9	—	—	—	8.4	8.0
Sugar present in respirometer	Lactose	Lactose	Glucose	Glucose	None	Lactose	Lactose	Glucose	Glucose	None	Lactose	Glucose	Lactose	Glucose	Glucose	Glucose	Glucose	None	Lactose	Lactose	Glucose
Time	Oxygen consumption in cubic millimeters per 10 mgm. of preparation																				
minutes																					
0–15	16	46	164	126	7	19	89	230	212	7	10	0	120	89	175	−3	199	16	27	46	112
15–30	13	41	183	125	19	23	87	196	189	8	3	1	148	86	170	1	205	19	7	42	108
30–45	17	42	196	117	7	22	83	201	196	12	4	6	166	80	167	5	193	16	30	52	116
45–60	15	33	183	119	7	19	90	175	167	13	0	3	165	88	161	8	199	19	10	50	112
60–75	11	35	167	102	10	26	95	161	157	10	2	1	173	79	154	8	185	14	29	56	117
75–90	16	33	172	110	13	28	91	160	157	9	−3	0	173	69	87	5	167	13	19	55	100
90–105	14	38	168	116	14	25	95	132	134	9	−1	1	168	78	139				36	62	121
105–120	15	39	156	102	12	28	96	103	107	7	4	6	166	76	118				28	60	103
Total oxygen consumption	117	307	1,359	917	89	190	726	1,358	1,319	75	19	18	1,279	645	1,171				192	423	889

that only a slight oxygen consumption by dried preparations occurs in lactose or glucose (samples 4 and 8). These results indicated that destruction of the oxidase system had occurred during the drying procedure, earlier work (Deere, Dulaney and Michelson, 1939) having demonstrated that lactase is not damaged by drying. Drying had not destroyed the oxidase system of the white strain (experiments 1 and 2). The most plausible explanation of the difference in behavior of the two strains appeared to be the difference in pH of the medium from which the

TABLE 2

Oxygen consumption of suspensions of Garrett red grown on lactose agar
Samples 10, 16 and 18 washed in $NaHCO_3$ to bring pH to 7.6
Substrate—0.1 M phosphate, pH 7.0, 0.5 per cent sodium chloride, and 0.5 per cent sugar

SAMPLE NUMBER...	4	7	8	10	16	18
Treatment of cells..	Dried	Dried	Non-dried	Dried	Dried	Non-dried
Weight of sample, mgm.............	5.6	5.6	—	4.7	4.4	—
Sugar present......	Lactose	Glucose	Glucose	Lactose	Glucose	Glucose
Time interval	Oxygen consumption in cubic millimeters					
minutes						
0–15	2	1	147	1	4	138
15–30	2	0	124	0	2	123
30–45	0	2	111	−2	4	117
45–60	1	1	101	1	−1	96
Total oxygen consumption	5	4	483	0	9	474

cells had been harvested. 24-hour growth on lactose agar produced a pH of about 5 for the red and above 7 for the white strain. It appeared unlikely that more than traces of organic acids would remain in the cells of the red strain during the process of washing, once in saline and twice in distilled water. This possibility, however, was now tested.

A 26-hour growth of Garrett red on 1 per cent lactose agar was harvested in saline as usual and the resulting suspension divided into two parts. One part was centrifuged without further

treatment. The second part was treated with an equal volume of M/10 sodium bicarbonate and then centrifuged. The supernatant fluid from the bicarbonate-treated sample had a pH of 7.6; the untreated sample had a pH of 4.9. Both samples were washed twice in distilled water in the usual manner and portions prepared for study of the effect of drying on oxygen consumption. The results (table 2) demonstrate a similar behavior of the non-treated and bicarbonate-treated samples. The non-dried samples consumed oxygen rapidly in glucose but the dried samples consumed negligible quantities of oxygen in lactose or glucose. Thus, it appeared that the oxidase system of the red strain was peculiarly susceptible to destruction by drying or that growth on a fermentable sugar produced a susceptibility to destruction, a susceptibility which was not altered by neutralization of the products of fermentation before drying.

We therefore tested the effect of drying on the oxidase activity of a plain agar culture of the red strain. A 23-hour growth of Garrett red gave a pH of 7.3, a reaction similar to that of a lactose agar culture of the white strain. The results (experiment 4, table 1) demonstrate the failure of the drying technique to paralyze the oxidase system of cells of the red strain grown in this manner. It appears then that growth of the red strain on a fermentable sugar is responsible for the susceptibility of its oxidase system to destruction by drying. It seemed unlikely that this behavior was peculiar or characteristic of only the red strain. This indicated the advisability of studying the behavior of suspensions of the white strain which had been grown on a fermentable sugar.

A 24-hour culture of Garrett white on 1 per cent glucose agar was treated in the usual manner and the effect of drying on the oxygen consumption of cells suspended in glucose was determined. The destructive effect of drying on the oxidase system of these cells is apparent (experiment 5, table 1). Our original plan of comparing the effect of drying on the lactase activity of the two strains could then be carried out. This required a dry preparation which retains oxidase activity which could be obtained with the red strain only by growing it on plain agar.

Study of a 25½-hour plain-agar culture of Garrett red revealed that drying doubled the oxygen consumption of cells suspended in lactose (experiment 6, table 1). Comparison of these results with those of experiment 1 and 2 demonstrates a similarity in the activating effect of drying on the lactase activity of both the white and red strains though the activation is more marked with the white.

It occurred to us that the increase in lactase activity which takes place on drying might be due to release of lactase from cells into the surrounding fluid. An experiment was designed to test this hypothesis. A 25-hour growth of Garrett white was harvested from 1 per cent lactose agar, the pH being 7.2. The cells were washed in the usual manner and resuspended in water. Four 1-cc. portions of the suspension were measured into Warburg flasks and set aside for respiration studies, after one of them, no. 7, had been dried by vacuum distillation. The weight of dry material was 7.1 mgm. Two 3 cc. portions of the suspension were measured into centrifuge tubes and used for preparation of extracts A and B.

The sample used for preparation of extract A was dried in a 50 cc. centrifuge tube by vacuum distillation, the weight of dry material being 21.3 mgm. After the dried sample had stood for 2½ hours, 3 cc. of 1.5 per cent sodium chloride in 0.3 M phosphate buffer were added to it. The cells were resuspended and 15 minutes later the sample was centrifuged. One cubic centimeter of the supernatant fluid represents the extract from 7.1 mgm. of dried cells and is designated as extract A.

A second 3-cc. portion of the original suspension was centrifuged and the supernatant fluid removed as completely as possible. Then 3 cc. of the phosphate-sodium chloride solution were added, the cells resuspended, and 5 minutes later the sample was centrifuged. One cubic centimeter of the supernatant fluid represents the extract from 7.1 mgm. of non-dried cells and is designated extract B.

For determination of the effect of these extracts on the oxygen consumption of cells, samples containing cells alone, extract alone, and cells in the presence of extract were prepared. Lac-

tose was added to all samples. The results of the experiment are presented in table 3.

Extract B (of non-dried cells) consumes no oxygen in the presence of lactose (sample 16). Addition of extract B to non-dried cells suspended in lactose (sample 8) results in no greater oxygen consumption than was observed in the absence of extract (sample 10). These facts are interpreted as indicating the absence of lactase in extract B.

TABLE 3

The lactase activity of an extract of dried cells of Garrett white

Substrate—0.1 M phosphate, pH 7.0, 0.5 per cent sodium chloride, and 0.5 per cent lactose

Extract A is an extract of dried cells. Extract B is an extract of non-dried cells

SAMPLE NUMBER...	8	16	10	4	7	18
Type of cells present..........	Non-dried	None	Non-dried	Non-dried	Dried	None
Extract present ...	B	B	None	A	None	A
Time interval	Oxygen consumption in cubic millimeters					
minutes						
0–15	6	−2	4	19	62	−3
15–30	11	2	14	17	62	−7
30–45	8	−1	5	18	65	0
45–60	11	0	12	17	64	−1
60–75	10	0	10	18	56	−2
75–90	11	0	10	18	56	0
Total oxygen consumption	57	−1	55	107	365	−13

Extract A (of dried cells) produces gas when added to lactose (sample 18). Since potassium hydroxide was present to absorb carbon dioxide, it seems probable that some hydrogen was produced. Addition of extract A to non-dried cells suspended in lactose (sample 4) results in an oxygen consumption 1.9 times that observed in the absence of extract (sample 10). We attribute these results to the presence of lactase in extract A.

DISCUSSION

The results reported here confirm previously reported findings that the difference in the two strains of *Escherichia coli-mutabile*

does not lie so much in lactase content as in lactase activity, the lactase of the white strain being inactive in uninjured cells. Several possible explanations of the presence of an inactive enzyme in a cell can be offered. One explanation is the presence of a specific enzyme inhibitor or antienzyme as postulated by Stewart (1926 and 1928). Our results do not contradict this hypothesis. In fact, our results could be explained easily if it could be shown that an antienzyme is, in reality, present. However, if this explanation is correct, the antienzyme is very labile for it is destroyed by antiseptics or by drying. We have considered trying to extract such a substance but, if it is present, its extreme lability has made the possibility of extracting it exceedingly remote.

Stewart believed that the variants were formed through the loss of this hypothetical antienzyme by growth on lactose. He assumed, but did not prove, the presence of lactase in the white form of *Escherichia coli-mutabile*. Lewis (1934) does not accept Stewart's view. Lewis believes he has demonstrated that the mutation of *Escherichia coli-mutabile* occurs continuously, irrespective of the medium.

A possible explanation which seems plausible to us is based on permeability of the bacterial cell. Is it not possible that the failure of the white strain to metabolize lactose is due to impermeability of the cell to lactose rather than to inactivity (antienzyme suppression) of its lactase? Search of the literature has revealed no answer to the question of the permeability of bacterial cell membranes to sugars. Lactase of this group of organisms is generally agreed to be intracellular. The methods which we have used for demonstration of lactase in *Escherichia coli-mutabile* undoubtedly injure the cells. Since uninjured cells of the white strain do not metabolize lactose, and since injury which is known to influence permeability renders them active towards lactose, it seems reasonable to suggest that the inability of the uninjured cells to metabolize lactose is due to their impermeability to lactose. We have demonstrated that significant amounts of lactase can be extracted from injured (dried) cells (table 3) but change in permeability of the cell to lactose may be of more consequence than change in permeability to lactase.

An hypothesis based upon permeability of the cells must explain the following facts:

1. The white strain contains lactase but is unable to ferment lactose. Impermeability of the cells to lactose would satisfactorily explain this fact.

2. Prolonged growth of the white strain in the presence of lactose results in the production of lactose-fermenting variants (reds). This transformation could be explained as a change from a cell impermeable to lactose to one permeable to it.

3. Treatment of cells of the white strain with antiseptics or simply drying them renders their lactase active. These treatments are injurious to the cell and cell injury is known to alter permeability. These facts fit well in the hypothesis.

4. Drying cells of the red strain increases their lactase activity as judged by their oxygen consumption in the presence of lactose. This fact can be reconciled with the permeability hypothesis on the assumption that the uninjured cells are difficultly permeable to lactose and that the drying process increases this permeability.

SUMMARY

Studies on the oxygen consumption of preparations of *Escherichia coli-mutabile* have demonstrated the following facts:

1. Lactase activity of preparations of the white strain does not depend on the presence of or preliminary treatment with an antiseptic. High vacuum drying suffices to increase the oxygen consumption in lactose to 3 to 6 times that observed with non-dried preparations.

2. Our previous demonstration of the necessity of the presence of lactose in the medium on which cells are grown in order to obtain high lactase activity is confirmed by the present study.

3. High-vacuum-dried preparations of the red strain harvested from lactose agar consume no oxygen when suspended in lactose. This behavior was shown to be due to inactivation of the oxidase system by the drying procedure.

4. Drying cells of the white strain which have been grown on glucose results in inactivation of their oxidase system. This phenomenon was observed with both the white and red strains

when they had been grown on fermentable carbohydrate and was not observed when they were grown in the absence of fermentable carbohydrate. The significance of this observation is not apparent.

5. Drying cells of the red strain which have been grown on plain agar yields a preparation which consumes more oxygen when suspended in lactose than does the corresponding non-dried preparation. In this case the increase in oxygen consumption is not as marked as with the white strain.

6. Extracts of dried cells of the white strain increase the oxygen consumption of non-dried cells suspended in lactose. A similar extract of non-dried cells does not have this effect.

An hypothesis of altered membrane permeability which attempts to explain the observed facts is presented.

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THE INTRODUCTION OF AGAR-AGAR INTO BACTERIOLOGY

ARTHUR PARKER HITCHENS

Army Medical School, Washington, D. C.

AND

MORRIS C. LEIKIND

Institute of the History of Medicine, Johns Hopkins University, Baltimore, Md.

Among fundamental developments in bacteriology the elaboration of solid culture substrates will always hold first place. With the introduction of a method for isolating microörganisms and growing them in pure culture, bacteriologists possessed the means for studying what Ferdinand Cohn (1872) called "Die kleinsten lebenden Wesen." Credit for the discovery of the solid medium technique is commonly accorded to Robert Koch, but, as is usual with discoveries of major importance in science, he had his fore-runners. Of course this detracts in no way from the glory of Koch's achievement; his genius lay in his ability to bring order out of chaos. Starting as it were with a box of miscellaneous beads, varying in size and shape, each bead a scientific fact, he found a thread on which the beads could be strung to form a perfect necklace.

There are, it seems, two types of workers in Science, the bead collectors and the bead stringers. Which of the two is more important must not be a question for discussion here. It is a fact, however, that bead collectors are more numerous than bead stringers and it is also an historical commonplace that the bead collectors are more obscure, less known to the world, than those geniuses who string necklaces. The historian of science must concern himself with both types of workers and their productions. The contributions of the obscure are sometimes as important as those of investigators in a more favorable position to be il-

luminated by the spotlight of fame.   Yet history does record the work of individuals, who, while generally unknown and unsung have made discoveries of lasting value.   As a foreword or "Golden Text" to his volume *Behind the Doctor*, Clendening (1933) quotes from Oliver Wendell Holmes that medicine learned "from a Jesuit how to cure agues, from a friar how to cut for the stone, from a soldier how to treat gout, from a sailor how to keep off scurvy, from a postmaster how to sound the Eustachian tube, from a dairymaid how to prevent smallpox, and from an old market-woman how to catch the itch-insect."

To this list we can now add a housewife and tell the story of how she helped her bacteriologist husband in his studies upon the microörganisms of the air and thereby achieved pure cultures. For many years it has been known to bacteriologists that agar-agar was introduced into bacteriological technique by a certain Frau Hesse.   But who Frau Hesse[1] was and how she came upon this important innovation has been until now generally unknown. In August of 1937 we came into the possession of additional facts concerning this discovery.   The story of Fannie Eilshemius Hesse is, we feel, of interest to all bacteriologists, but especially to American students of this branch of science.   Following the publication of his brief memorial notice in the Zentr. f. Bakt., 1935, we wrote to Professor Edgar von Gierke (1935) asking if he would put us on the track of information we had been seeking for many years.   He kindly referred our letter to Dr. Friedrich Hesse, a surgeon now living in Dresden, Germany. He is the son of Frau Hesse and graciously provided us with the facts upon which our story is based.   We have also received from him portraits of his parents, here reproduced for the first time.   For these facts and photographs, bacteriologists in general owe a debt of gratitude to Dr. Friedrich Hesse.

Frau Hesse was born Fannie Eilshemius in 1850 in a locality now incorporated in Jersey City in the state of New Jersey.

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[1] There are two brothers of Frau Hesse still living in New York City, Mr. Henry and Mr. Louis Eilshemius.   The latter is a well known artist, his work being the subject of an illustrated paper published in the October (1937) number of the *Magazine of Art*.   He has often abbreviated his name to Elshemus.

At that time this community was known as Laurel Hill.   Her
father had immigrated here from Germany in 1818.   We know
little of her early life.   In 1874 she went abroad, traveling over
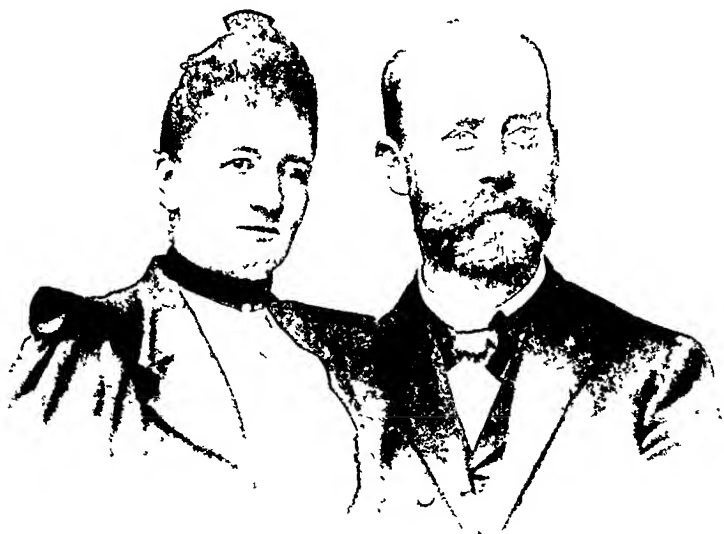
FRAU FANNY EILSHEMIUS AND DR. WALTHER HESSE

Europe and finally coming to Germany.   It was there she met
Walther Hesse, then a district physician in Schwartzenberg,
Saxony.   Young Hesse was more than a mere country doctor
and health officer.   He was aware of the new scientific horizons

appearing in the work of Pasteur, Koch, Ferdinand Cohn and many others, for in the winter of 1881–82 he became a student of the then new science of Bacteriology under Robert Koch in the laboratories of the *Kaiserliche Gesundheitsamt*, in Berlin. Many problems were pressing for solution in those early days and although the most important were those dealing with the bacteria causing infectious disease, the problem of the general distribution of these organisms was not neglected. Hesse (1884) began to study the bacterial content of the air.[2] These studies were continued at his home in Schwartzenberg after his half-year period of residency in the Imperial Health Office. It was in his home, now also his laboratory, that the momentous discovery was made. To understand the importance of this new technical achievement we must review briefly a few facts regarding the historical development of pure culture methods.

As we now know so well from the work of Dobell (1932) and the translation of Cohen (1937), bacteria had been seen and described by Leeuwenhoek just a little over two hundred years before Hesse began his research. During most of the intervening period observers had been content to study them as they were found in nature. Until the time when Pasteur (1857) began his researches on fermentation few individuals had seemed to be interested in whether bacteria could be grown or not. Except to a few botanists, especially Ferdinand Cohn (1872), the problem of the classification of species and genera was of little moment. Most students agreed with Linnaeus (1763) that bacteria belonged in the class *Chaos;* they were little advanced beyond the early classification made by Otho Fridericus Müller (1773). With the studies of Pasteur (1857), Cohn (1872), and Koch (1881, 1882) a new era began. Pasteur, although no systematist, demonstrated the physiological specificity of bacteria. In his studies on fermentation he showed that each type of the organisms which he called by so many different

[2] The results of this work were finally published in vol. II (1884) of the Contributions from the Gesundheits-amt. The paper is illustrated by three double page chromolithographs. The original paintings for these were made by Frau Hesse. They are in every way excellent.

names was characterized by and responsible for specific physiological activities.   In order to prove his contention that specific organisms could bring about alcoholic, lactic or butyric fermentations he devised simple nutrient fluids of known composition to demonstrate these reactions.   By transferring minute quantities of fluid containing bacteria from flask to flask he was able to obtain relatively pure cultures.   At least they were pure on the basis of the fermentations brought about.   There were various attempts to modify his medium and many workers, among them Klebs (1873), Lister (1878), and Salomonsen (1876), attacked the problem of growing pure cultures, with varying degrees of success.   The great stumbling block was that they were all working with fluid culture media and the difficulties were almost insuperable.   But another group of workers, especially those interested in fungi, were attacking the problem from a more productive angle.   As related so dramatically by Harrison (1924) and echoed by Reid (1936) people had for centuries been terrified and awed by epidemics of "blood spots" miraculously appearing on food stuffs, especially the holy wafers.   In 1819 an extensive and persistent epidemic of "bloody bread" broke out in Padua.   An official investigation was made and one of the investigators, Bartolomeo Bizio, recognized the fungus nature of the blood-red growth.   He isolated it from some corn porridge (polenta) and carried out a series of successful transfers to other farinaceous materials.   Bizio (1823) named the organism *Serratia marcescens: Serratia*, in honor of Serafino Serrati, who was the first to run a steamboat on the Arno, the species name signifying putrefaction.   Bizio is not heard of further but we may date the first attempts to grow bacteria on solid culture media from his experiments.   Observations were made concerning the more or less accidental growth of bacteria on various organic solid media in the years that followed, but there were none planned with definite and serious purpose until we come to the work of Schroeter (1872), a pupil of Ferdinand Cohn.   The paper that he published in 1872 on the chromogenic bacteria is a landmark.   He was able to separate various chromogens from each other by growing them on solid media such as potato, coagu-

lated egg white, starch paste and meat. Schroeter found that on these solid substrates his pigment producers often appeared as isolated spots of color. He then learned that all organisms in a single spot or colony were alike. When he transferred organisms from a single colony to fresh media he was able to grow the same organisms and, by repeated transfer, to continue the process indefinitely. He had there the secret of pure culture study but technical difficulties stood in the way of the universal application of his method. The difficulties were due to the presence of nonchromogenic bacteria, especially when the unpigmented colonies closely resembled the color of the medium. It was only a few years later that Koch (1881) entered the field and his genius promptly solved these disturbing problems. He recognized that one must have a substrate which was at once solid, transparent and sterile. What he sought was a universal culture medium, but he soon recognized the impracticability of this quest. He then turned his attention to finding a suitable jelly that could be incorporated with a nutrient fluid. His most promising material was gelatin which the mycologists, among them Vittadini (1850), had been using, for 30 years. With the aid of this medium and his plating and dilution method, Koch (1881) revolutionized bacteriological technique. Isolating pure cultures was, in comparison with the older techniques, enormously simplified. However, the way was not yet completely cleared. Gelatin was not quite the ideal jellifying medium required. It possessed two undesirable qualities. First, it was liquefied by certain organisms. This characteristic, while of value in the identification of a lytic ferment, ruined the medium for the purposes of isolating pure cultures. The second defect arose from the fact that gelatin is not solid but fluid at 37°C. incubator temperature. It was satisfactory for organisms cultivable at room temperatures—22°C., or below—but not for pathogens requiring blood heat.

It was with these difficulties that Walther Hesse and his wife, Fannie, wrestled. Fannie was not merely the "Hausfrau," she was also the technician and the artist illustrator in the home

and laboratory of Dr. Hesse. She cooked not only the soup for her family but also bouillon for her husband's bacteria. In the studies of atmospheric bacteria made by Dr. Hesse (1884), tubes lined with gelatin were used. Measured quantities of air were aspirated through these tubes and from the colonies of bacteria developing on the lining medium the numbers and kinds of microbes in the air were listed. But the maddening liquefaction of gelatin ruined many of the experiments and finally Hesse began to seek new solidifying agents. At this point Frau Hesse became an historic figure. She suggested the use of agar-agar which she had been using for years in her kitchen in the preparation of fruit and vegetable jellies. While yet in America she had received the recipe from her mother; her mother in turn had obtained the formula from some Dutch friends who had formerly lived in Java. In the East Indies, where the source of agar-agar, Japanese seaweed (*Gelidium corneum*), also abounds, this curious material had been used for generations as a jellifying agent and as a thickening for soups (Smith, H. M., 1905).

We can imagine the elation of Dr. Hesse when he set up and studied his tubes lined with his wife's new medium and found that his troubles were at an end. Now he could prepare a substrate, solid, transparent and sterile, which would retain its consistency at all temperatures at which bacteria could grow and which, furthermore, would not be liquified by any of the organisms he encountered in his studies. Without delay the discovery was communicated to Robert Koch by letter, probably late in 1881. Koch recognized its value and made it his own. In 1882, in his now classic preliminary note on the tubercle bacillus, Koch made what is the first printed reference to the use of agar—just one short sentence for a technical improvement so fundamental and epoch making. No formal paper was ever published.

Thus did a modest housewife perform a service to science and to humanity. When she died in 1934, few bacteriologists knew of her death, few perhaps that she had ever lived. Lesser innovations and discoveries are commemorated with the name of

the discoverer. Could not "plain agar" from now on be designated as "Frau Hesse's medium?" Her contribution to bacteriology makes her immortal.

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NICOTINIC ACID AND THIAMIN HYDROCHLORIDE AS GROWTH-PROMOTING FACTORS FOR BRUCELLA

GRACE P. KERBY

From the Brucellosis Laboratory, Clayton Foundation for Research, Nix Professional Building, San Antonio, Texas

Nicotinic acid and vitamin B-1 (thiamin hydrochloride) have aroused considerable interest as growth-promoting substances for bacteria in artificial media. Mueller (1937), working with the diphtheria bacillus, found that nicotinic acid accounts for a part of the growth-promoting activity of tissue extracts for that organism, its maximal effect being exerted "at a concentration of about 1 mgm. per liter of medium but varying somewhat with the composition of the control medium." Knight (1937, a and b) demonstrated the necessity of nicotinic acid and its amide for the growth of *Staphylococcus aureus*, his findings being confirmed later by Landy (1938). Knight (1937 c) also found that, in addition to nicotinic acid, vitamin B-1 is required by the staphylococcus. In the present study an attempt has been made to determine (1) if these substances, when added to routine media, will enhance the growth of *Brucella* and (2) if so, what concentrations will produce maximal results.

Bacto Tryptose Agar has been used as the basic medium. Two recently isolated strains of *Brucella abortus* (*B. abortus* RV-3 and 1033), 4 stock strains of *B. abortus* (*B. abortus* G-1153, 456, 161 and 230) and one stock strain of *Brucella melitensis* were employed in the study. A uniform inoculum of 0.05 cc. of a suitably diluted broth culture was used throughout for each plate.

Nicotinic acid was added to the medium in amounts ranging from 4 to 40 mgm. per liter, and thiamin hydrochloride in a range of from 0.01 to 100 mgm. per liter. Studies of the two
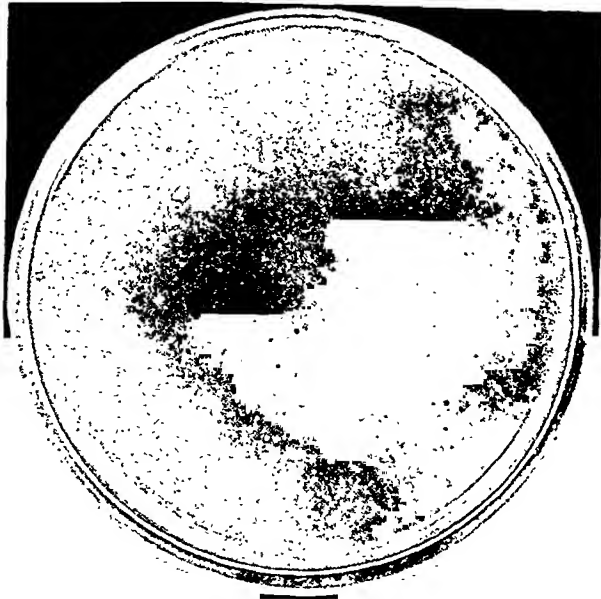
Fig. 1. Growth of Brucella abortus 456 on Bacto Tryptose Agar Control

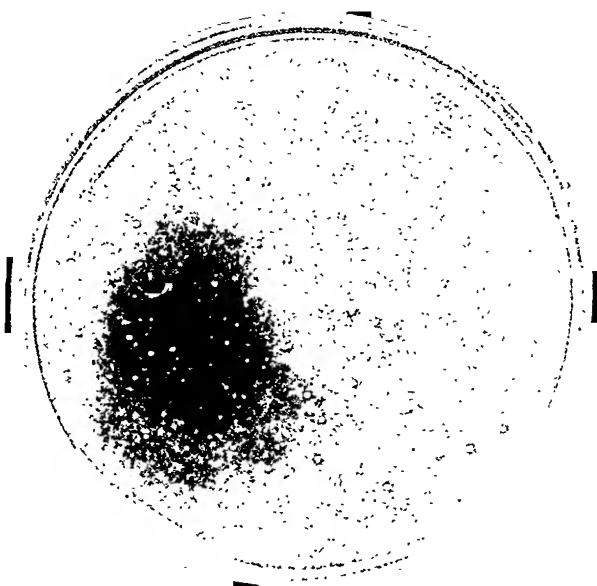Plates were incubated 48 hours at 37°C. and 18 hours at room temperature before photographing.

Fig. 2. Growth of Brucella abortus 456 on Bacto Tryptose Agar Containing 30 mgm. Nicotinic Acid per Liter of Medium

Plates were incubated 48 hours at 37°C. and 18 hours at room temperature before photographing.

FIG. 3. GROWTH OF BRUCELLA ABORTUS 456 ON BACTO TRYPTOSE AGAR CONTAINING 25 MGM THIAMIN HYDROCHLORIDE PER LITER OF MEDIUM

Plates were incubated 48 hours at 37°C. and 18 hours at room temperature before photographing

FIG 4. GROWTH OF BRUCELLA ABORTUS 456 ON BACTO TRYPTOSE AGAR CONTAINING 30 MGM NICOTINIC ACID AND 25 MGM THIAMIN HYDROCHLORIDE PER LITER OF MEDIUM

Plates were incubated 48 hours at 37°C. and 18 hours at room temperature before photographing.

substances separately indicated that the optimal concentration of nicotinic acid was 30 mgm. per liter of medium and of thiamin hydrochloride, 25 mgm. per liter.

Growth of all strains of *B. abortus* was enhanced, but to different degrees, by either nicotinic acid or thiamin hydrochloride. Thus, stock strains of *B. abortus* 456 and 161 grew better on the nicotinic acid medium, while stock strain *B. abortus* 230 grew better in the vitamin B-1 medium. Two recently isolated strains of *B. abortus* and stock strain *B. abortus* G-1153 grew best on the combination of nicotinic acid and thiamin hydrochloride. All strains of *B. abortus* showed as good or better growth on media containing both substances as they did when the materials were used separately.

The growth-promoting effect of these substances was noted mainly in the speed of initial growth; on the enriched media, colonies could be counted fully 24 hours before those on control plates. Above and below the optimal concentrations mentioned, the effect was diminished or lost. Ultimate size of colonies was also somewhat greater; but increase in number of colonies, while frequently observed, could not be considered significant because of the technical impossibility of insuring strictly uniform inocula.

In contrast to the effect of nicotinic acid and thiamin hydrochloride on the growth of *B. abortus*, the single stock strain of *B. melitensis* which was studied was inhibited slightly by the addition of either material. Whether this effect is typical of all melitensis strains must await further study.

Strains of *Brucella suis* were not investigated.

SUMMARY

The growth of *Brucella abortus* is enhanced by the addition of nicotinic acid and thiamin hydrochloride to Bacto Tryptose Agar in concentrations of 30 and 25 mgm., respectively, per liter of medium. Growth of certain strains is enhanced more by one than the other of these substances, but all strains grow well when both materials are added. For routine use, the combination seems advisable.

The single stock strain of *Brucella melitensis* studied was inhibited somewhat by either factor. *Brucella suis* was not investigated.

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THE OXIDATION OF ASCORBIC ACID AS INFLUENCED BY INTESTINAL BACTERIA[1,2]

WILLIAM B. ESSELEN, JR. AND JAMES E. FULLER

Department of Bacteriology and Physiology, Massachusetts State College, Amherst, Mass.

INTRODUCTION

Stepp and Schroder (1935), and Stepp (1936), reported that certain strains of intestinal bacteria, particularly *"B. coli communis"* and *"B. paratyphosus B"*, were capable of destroying ascorbic acid (vitamin C), while other intestinal bacteria were without action on this substance. They discussed the possibility that some cases of scurvy might be caused by the destruction of ascorbic acid by bacteria in the upper intestinal tract before the acid could be absorbed. Marin (1936) described a clinical case of scurvy which he believed had been caused by an "infection of *B. coli* and *B. paratyphosus B* in the upper intestinal tract."

Prompted by the above work the investigation here reported was carried on to study the action of normal intestinal bacteria on ascorbic acid, and factors influencing such action. In the event that certain kinds of intestinal bacteria may destroy ascorbic acid while others do not, it would be advisable from the standpoint of proper human nutrition to attempt to control these offending organisms in the intestinal tract. Such an alteration of intestinal flora possibly might be brought about by diet. However, this investigation in itself is concerned with an *in vitro* study of certain kinds of intestinal bacteria and their action on ascorbic acid under different conditions.

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[1] Contribution no. 316 of the Massachusetts Agricultural Experiment Station, Amherst, Mass.

[2] Based on portions of a thesis presented by William B. Esselen, Jr., April, 1938, to the Faculty of the Graduate School of the Massachusetts State College in partial fufillment of the requirements for the degree of Doctor of Philosophy.

The literature contains very little information pertaining to bacteria and ascorbic acid. Einhauser (1937) found that patients with achlorhydria were not easily saturated with ascorbic acid administered by mouth, and he attributed this effect to the destructive action of an acid condition and of bacteria in the stomach and upper part of the intestine. This work corroborates that of Stepp and of Marin mentioned above. Gagyi (1936), and Gagyi and Ujsaghy (1936), found that certain bacteria, particularly the more virulent pathogens, possess a capacity for destroying ascorbic acid, and that the inability of these organisms to grow in a 0.2 per cent solution of this substance and their ability to destroy it run parallel. Hou (1936) reported that the destruction of vitamin C experimentally in distilled water or in tap water was caused partly by microorganisms.

The incorporation of ascorbic acid in culture media for the cultivation of anaerobes has been investigated to a limited extent. Ehrismann (1935) found that ascorbic acid in a dilution of 1:1000 favored the growth of obligate anaerobes even under aerobic conditions. Kligler and Guggenheim (1938) found that vitamin C used in culture media for *Clostridium welchii* reduced the oxidation-reduction potential of the medium sufficiently so that this organism would grow even in the presence of air or of oxygen. They also observed that the loss of ascorbic acid was less in a growing culture of *C. welchii* than in sterile control media.

Kligler, Leibowitz, and Berman (1937), in a study of the effect of ascorbic acid on toxin production by *Corynebacterium diphtheriae* in culture media, found that ascorbic acid was rapidly oxidized in culture media at a temperature of 30° to 37°C., but that it remained unoxidized longer in the presence of bacterial growth. They suggested that ascorbic acid may destroy by an oxidation-reduction reaction the toxin produced by this organism, and that the mechanism is similar to that of the toxin-destroying action of aldehydes in general.

Tkachenko (1936) appears to be the only one who has reported

studies on the ability of bacteria to reduce dehydroascorbic acid to ascorbic acid. He found that this conversion took place in cultures of *Lactobacillus bulgaricus*, *L. acidophilus*, and *L. leichmanni*.

Some work has been done relative to the effect of microbial growth on the vitamin C content of foods. Lepkovsky, Hart, Hastings, and Frazier (1925) found that *Streptococcus lactis* had no effect on the vitamin C content of orange and tomato juices. Bifano and Servazzi (1935) infected lemons with *Penicillium digitatum* and found that their vitamin C content was the same as that of non-infected lemons.

Esselen (1938) found that a number of different intestinal bacteria exerted a "protective action" on the oxidation of ascorbic acid. He suggested that this "protective action" might be correlated with bacterial growth activities. Kendall and Chinn (1938) reported an investigation on the decomposition of ascorbic acid by bacteria. As a result of their work it would appear that specific strains of bacteria, rather than bacterial "species" are able to ferment ascorbic acid. They also reported that certain bacteria growing in an ascorbic acid medium exhibit a "protective action," that is, actually retard the oxidation of ascorbic acid. They found, too, that glucose exerts a definite sparing action upon the fermentation of ascorbic acid by bacteria.

Thus, a review of the literature indicates that bacteria may be classified into three groups as regards the stability of ascorbic acid in their presence, namely: (1) those organisms which destroy ascorbic acid; (2) those which protect ascorbic acid from oxidation; and (3) those which have no influence on this substance.

EXPERIMENTAL WORK

Methods

Throughout this investigation cultural methods described below were employed in the study of the action of bacteria on ascorbic acid. The media used were physiological saline (0.85 per cent sodium chloride), and nutrient broth of the following composition:

"Bacto" beef extract.................................   3.0 grams
"Bacto" peptone....................................   5.0 grams
Carbohydrate (when used)........................... 10.0 grams
Distilled water.................................... 1000.0 ml.
Reaction... pH 6.3 to 6.5

This medium was prepared and distributed in 50 ml. test tubes containing 30 ml. of medium, or in 250 ml. Erlenmeyer flasks containing 100 ml. of the medium, and autoclaved for 20 minutes at 15 pounds pressure. One-milliliter portions of a 24-hour nutrient broth culture of the organism studied were used for inoculation. An incubation temperature of 37°C. was used at all times. Pure crystalline ascorbic acid was employed, and the desired amount added to the medium in aqueous solution.

The ascorbic acid content of the medium was determined at the beginning and end of each experiment by a modification of the 2,6-dichlorophenolindophenol dye method of Tillmans as described by Bessey and King (1933). The actual method employed throughout this study consisted in placing 20.0 ml. of the culture mixture into a 50 ml. flask and adding 5.0 ml. of glacial acetic acid (sufficient to lower the pH value of the titration mixture below 2.0). The acidified solution was titrated immediately with a standardized solution of the 2,6-dichlorophenolindophenol dye. The titration was completed within two minutes in each case. Determinations were made in duplicate or in triplicate, and good checks were obtained. For convenience, all data are reported in terms of milligrams of ascorbic acid per 20.0 ml. of medium, and as the per cent of loss of ascorbic acid during the incubation period. In studying the effect of bacteria on ascorbic acid different incubation periods were used, and the ascorbic acid content of the mixture was determined at the beginning and at the end of each incubation period. Thus, it was possible to calculate the per cent loss of ascorbic acid as influenced by the various factors studied. Throughout most of this work much smaller concentrations of ascorbic acid have been used than were used by previous workers, because, under actual conditions existing in the intestinal tract, ascorbic acid is present in only relatively small amounts.

From a preliminary study it was found that ascorbic acid is

rapidly oxidized in uninoculated culture media. Consequently, it was decided to use a five-hour incubation period in studying the influence of bacteria on ascorbic acid because, on longer incubation, the ascorbic acid itself would be practically all destroyed without bacterial influence and it would be difficult to interpret the results as to the influence that bacteria might have on this oxidation. It was also found that, throughout a range of pH values from 3.85 to 9.40, the degree of acidity had no significant influence on the rate of oxidation of ascorbic acid under the conditions of the experiment. Thus, it would seem that the change in the reaction of the medium due to bacterial action would not be a factor in influencing the oxidation of ascorbic acid because this substance is rapidly oxidized throughout the normal pH range of bacterial growth.

The oxidation of ascorbic acid in nutrient and in carbohydrate broth inoculated with various intestinal bacteria

As a preliminary step in studying the destruction of ascorbic acid as influenced by bacterial growth, eleven strains of *Escherichia coli*, and one strain each of *Aerobacter aerogenes, Salmonella enteritidis, Salmonella pullorum, Salmonella aertrycke, Eberthella typhosa, Bacillus subtilis*, and *Proteus vulgaris* were studied with reference to their influence on ascorbic acid in nutrient broth, carbohydrate broth, and physiological saline by the method described above. The carbohydrates employed were glucose, galactose, lactose, maltose, sucrose, dextrin, and starch. The pH value of each culture was determined at the end of the incubation period by means of a Beckman pH meter. The data obtained are presented in table 1.

Ascorbic acid determinations on control cultures of the organisms with no ascorbic acid present gave no indication that ascorbic acid or ascorbic acid-like substances were synthesized by the bacteria. In no instance did the bacteria cause any greater destruction of ascorbic acid than that which occurred in the uninoculated control medium. Most of the organisms, particularly the *Aerobacter* and *Escherichia* strains, exerted a pronounced inhibitory action on the oxidation of ascorbic acid.

TABLE 1

Final pH of the medium and loss of ascorbic acid by oxidation in inoculated nutrient broth, carbohydrate broth, and physiological saline

Incubation five hours at 37°C.

ORGANISM	NUTRIENT BROTH		GLUCOSE BROTH		GALACTOSE BROTH		LACTOSE BROTH		MALTOSE BROTH		SUCROSE BROTH		DEXTRIN BROTH		STARCH BROTH		PHYSIOLOGICAL SALINE, PER CENT LOSS
	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	
Control	6.3	85	5.8	80	5.8	78	4.7	89	5.9	88	5.8	86	5.8	84	6.3	88	47
A. aerogenes (A101)	5.9	51	4.6	37	4.9	65	5.1	71	5.4	67	4.7	71	5.4	54	6.3	69	29
E. coli (Crooks)	5.7	56	4.4	28	5.1	28	5.3	51	5.4	40	5.6	66	5.3	35	6.3	72	31
E. coli (A1)	5.3	64	4.7	28	5.3	31	5.4	65	5.8	54	5.8	66	5.6	40	6.1	72	31
E. coli (A2)	5.7	68	4.7	30	5.2	42	5.8	72	5.8	71	5.8	74	5.7	42	6.3	73	35
E. coli (A3)	5.6	75	4.6	32	5.3	47	5.4	63	5.7	65	5.8	74	5.6	54	6.3	81	25
E. coli (A4)	5.7	67	4.6	18	5.3	42	5.3	71	5.7	52	5.8	74	5.6	48	6.1	71	33
E. coli (A11)	5.5	63	4.6	26	5.2	37	5.5	61	5.8	49	5.8	64	5.7	38	6.3	64	29
E. coli (A12)	5.7	67	4.7	26	5.2	38	5.4	61	5.8	61	5.8	63	5.7	37	6.0	73	27
E. coli (A20)	5.8	49	4.6	31	5.3	34	5.4	60	5.8	56	5.8	61	5.8	37	6.4	63	11
E. coli (A21)	5.5	62	4.8	31	5.6	33	5.4	70	5.8	58	5.8	61	5.8	29	6.2	64	27
E. coli (A25)	5.7	63	4.8	33	5.4	41	5.5	67	5.7	73	5.1	64	5.6	40	6.2	71	
E. coli (A30)	5.3	69	4.7	49	5.2	22	5.5	74	5.7	69	5.0	68	5.5	45	6.0	75	
S. pullorum	5.2	82	5.9	80	6.0	75	6.2	87	6.2	79	6.0	80	6.0	80	6.4	79	
S. aertrycke	5.7	57	4.8	58	5.4	34	6.2	88	6.0	65	6.0	66	5.9	42	6.3	56	
S. enteritidis	5.8	70	4.8	50	5.3	37	6.1	88	5.9	54	6.0	66	5.8	36	6.1	63	
E. typhosa	6.0	78	5.0	50	5.4	66	6.1	83	5.8	79	5.5	71	5.8	62	6.3	80	47
P. vulgaris	5.9	65	5.8	65	6.0	66	6.2	67	6.2	71	6.1	80	5.9	47	6.3	77	38
B. subtilis	5.8	80	6.0	83	6.0	73	6.1	70	6.2	85	6.1	78	6.1	81	6.2	81	42

The cultures of those organisms which grew most rapidly exerted the strongest inhibitory action on the oxidation of ascorbic acid. The presence of a readily fermentable carbohydrate in the medium still further enhanced the ability of the microörganisms to inhibit the oxidation of ascorbic acid. In physiological saline the organisms appeared to exert a slight inhibitory action on the oxidation of ascorbic acid in some cases, but, as with the nutrient broth, in no instance did they cause any destruction of this substance. It is also evident from table 1 that the degree of acidity produced by the bacteria under observation is not a factor in the ability of the bacteria to inhibit the oxidation of ascorbic acid. There appears to be no correlation between the acidity produced by an organism and its ability to inhibit the oxidation of ascorbic acid.

Influence of relative number of bacteria and their stage of growth on the oxidation of ascorbic acid

A test was conducted to determine the influence of the size of inoculum on the loss of ascorbic acid by *Escherichia coli, Aerobacter aerogenes,* and *Proteus vulgaris.* From table 2 it may be seen that, in general, as the size of the inoculum was increased the oxidation of ascorbic acid was decreased. These results indicated that the inhibitory effect of bacteria on the oxidation of ascorbic acid increased with the increase in the number of bacteria present. In the event that such is the case, older but still active cultures of bacteria which would contain large numbers of organisms should inhibit the oxidation of ascorbic acid to a greater extent than the relatively young cultures so far studied.

A series of experiments were carried out to test this hypothesis. Ascorbic acid was added to 5, 17, 24, 48, and 72-hour nutrient broth and glucose broth cultures of the organisms previously used. After five hours the loss of ascorbic acid was determined. The data so obtained are presented in table 3. The ability of all of the strains studied to inhibit the oxidation of ascorbic acid increased with the age of the cultures up to 24 hours, but beyond this time the cultures of the organisms appeared slowly to lose this property. The cultures exhibited their most marked inhib-

itory action towards ascorbic acid oxidation when they were in the stages of growth in which the maximum number of viable organisms was present in the medium (Jordan and Falk (1928)). Organisms in the glucose broth developed the power to inhibit the oxidation of ascorbic acid sooner than did duplicate cultures in nutrient broth.

Apparently, then, the actively growing bacteria bring about some change in the media which tends to inhibit the oxidation of

TABLE 2

Influence of the quantity of inoculum on the loss of ascorbic acid in nutrient broth incubated at 37°C. for five hours

ORGANISM	QUANTITY OF INOCULUM	ASCORBIC ACID PER 20 ML. NUTRIENT BROTH	LOSS IN FIVE HOURS
	ml.	mgm.	per cent
Control (start).	None	0.65	
Control (5 hours). .	None	0.07	89
	0.1	0 07	89
E. coli (Crooks) .	1.0	0.20	69
	5.0	0.45	30
	10.0	0.35	46
	0.1	0.10	85
A. aerogenes	1.0	0.15	77
	5.0	0.47	28
	10.0	0.60	8
	0.1	0.07	89
P. vulgaris...................	1.0	0.08	88
	5.0	0.08	88
	10.0	0.08	88

ascorbic acid. Several possibilities present themselves in explanation of what happens during bacterial growth which might inhibit the oxidation of ascorbic acid, namely: (1) the formation of un-ionized copper complexes with protein decomposition products, by which the catalytic power of small amounts of copper naturally present (Dunham (1938)) might be destroyed (Barron, Barron, and Klemperer (1936)); (Ettisch, Sachsse, and Beck (1931)); (2) a change in the oxygen tension of the medium and its saturation with other gases given off as a result of bacterial

TABLE 3

Influence of the age of culture on the rate of oxidation of ascorbic acid in nutrient broth

ORGANISM	AGE OF CULTURE*	PER CENT LOSS OF ASCORBIC ACID IN 5 HOURS AT 37°C.†	
		Nutrient broth	Glucose broth
	hours	*per cent*	*per cent*
A. aerogenes (A101)	0	56	36
	5		8
	17	8	3
	24	0	0
	48	24	
	72	17	
E. coli (Crooks)	0	37	19
	5	36	
	17	19	7
	24	0	0
	48	15	
	72	22	
E. coli (A2)	0	63	11
	5	48	
	17	17	6
	24	0	0
	48	17	
	72	20	
E. coli (A25)	0	67	37
	5	45	
	17	37	3
	24	0	14
	48	22	
	72	31	
P. vulgaris	0	85	80
	5	49	17
	17	11	3
	24	0	0
	48	11	
	72	26	
B. subtilis	0	90	88
	5	47	33
	17	20	0
	24	32	0
	48	23	
	72	69	

* Age of culture when ascorbic acid was added.

† Media contained 0.87 mgm. of ascorbic acid at start of incubation periods.

growth activity; and (3) the production of bacterial metabolic products which might prevent the ascorbic acid from being oxidized.

The influence of the concentration of ascorbic acid on its rate of oxidation and on the growth of bacteria

Several series of flasks each containing 100 ml. of glucose broth were prepared so as to contain ascorbic acid in concentrations approximating 1.0, 0.1, 0.01, and 0.001 per cent respectively. Inoculums of three strains of *Escherichia coli* and one strain each of *Aerobacter aerogenes*, *Proteus vulgaris*, and *Bacillus subtilis* were added separately to the different series of flasks for 18 hours. After incubation, plate counts were made from each flask in order to determine the influence of the concentration of ascorbic acid on the growth of the bacteria. Ascorbic acid determinations were also made so that the per cent of loss of this substance as influenced by its concentration and the presence of bacterial growth in the medium could be calculated. These data are summarized in table 4. From this information it is quite evident that bacterial growth is inhibited as the concentration of ascorbic acid in the medium is increased above certain concentrations, depending upon the kind of bacteria employed. However, with the slower growing organisms such as *Proteus vulgaris*[3] and *Bacillus subtilis*, concentrations of ascorbic acid up to 0.01 per cent and 0.1 per cent respectively appeared to stimulate growth.

At the beginning of these experiments the influence of the concentration of ascorbic acid on the pH values of the media was measured and was found to be as follows:

Glucose broth without ascorbic acid............................ pH 6.31
Glucose broth plus 0.001 per cent ascorbic acid.............. pH 6.31
Glucose broth plus 0.01 per cent ascorbic acid................ pH 6.08
Glucose broth plus 0.1 per cent ascorbic acid................. pH 4.60
Glucose broth plus 1.0 per cent ascorbic acid................. pH 3.65

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[3] While *Proteus vulgaris* is generally considered to be a rapidly growing organism, the strains employed by the author grew slowly in comparison with *Escherichia coli*.

TABLE 4

The influence of concentration of ascorbic acid upon its rate of oxidation and upon bacterial growth in glucose broth at 37°C.

ORGANISM	ASCORBIC ACID PER 20 ML. MEDIA	LOSS OF ASCORBIC ACID IN 18 HOURS	NUMBER OF ORGANISMS PER ONE ML. OF MEDIA AFTER 18 HOURS
	mgm.	per cent	
Control (start)	181.93		
	18.13		
	1.75		
	0.11		
Control (18 hours)	169.93	7	
	7.93	56	
	0.08	95	
	0.01	00	
A. aerogenes (A101)	181.90	0	10,500
	15.93	12	100,000,000
	1.37	22	100,000,000
	0.11	0	130,000,000
	None		130,000,000
E. coli (Crooks)	139.93	23	13,500
	17.73	2	23,400,000
	1.61	8	80,000,000
	0.09	18	170,000,000
	None		250,000,000
E. coli (A2)	181.00	0	30,000
	15.33	14	14,400,000
	0.93	47	200,000,000
	0.05	55	132,000,000
	None		250,000,000
E. coli (A25)	179.93	1	20,000
	16.73	8	12,000,000
	0.79	55	50,000,000
	0.03	73	86,000,000
	None		100,000,000
P. vulgaris	141.93	22	20,000
	13.13	28	7,800,000
	0.54	69	70,000,000
	0.02	82	128,000,000
	None		20,000,000
B. subtilis	169.93	7	1,600
	18.10	0	4,200,000
	0.27	85	43,000,000
	0.01	99	150,000,000
	None		800,000

The above information would suggest that the inhibition of bacterial growth observed as a result of increasing the concentration of ascorbic acid may be due to the increased hydrogen ion concentration of the media produced by the ascorbic acid.

The production of carbon dioxide by bacteria as a factor influencing the stability of ascorbic acid

In the event that the observed inhibitory action of bacteria on the oxidation of ascorbic acid as reported above might have been due to gases produced by the organisms, the influence of carbon dioxide and of hydrogen on ascorbic acid oxidation was studied. Eighteen different bacterial strains were inoculated into flasks of nutrient broth to which ascorbic acid was added according to the technique previously employed. The flasks were incubated in anaerobe jars for five hours at 37°C. in atmospheres of carbon dioxide and of hydrogen respectively. The results are presented in table 5. In comparing these results with those in table 1 it may be seen that, in general, the oxidation of ascorbic acid in an atmosphere of hydrogen is similar to that which takes place in nutrient broth under aerobic conditions. The carbon dioxide exerted an inhibiting effect on the oxidation of ascorbic acid to a degree parallel to that exerted by the bacteria when they were cultured in nutrient broth containing a readily fermentable carbohydrate. Thus, it would seem that the protective action of the bacteria on ascorbic acid may be due, at least in part, to the carbon dioxide which they produce. This possibility was investigated by measuring the quantities of total dissolved gas and of dissolved carbon dioxide which were present in the cultures as a result of bacterial metabolism. The manometric method of Van Slyke and Neill, as described by Hawk and Bergeim (1931) was employed for the measurement. The quantities of total gas and of carbon dioxide produced by the bacteria in the cultures were compared with the degree of oxidation of ascorbic acid in the medium as influenced by the bacteria present.

The results are given in table 6, from which it may be seen that the organisms which significantly inhibited the oxidation of

ascorbic acid also produced considerable carbon dioxide and total gas in comparison with those which did not retard the oxidation of ascorbic acid. Although these data show a correlation between carbon dioxide production by bacteria and their ability to inhibit

TABLE 5

Influence of atmospheres of carbon dioxide and hydrogen on the oxidation of ascorbic acid in nutrient broth inoculated with different intestinal bacteria in five hours at 37°C.

ORGANISM	IN ATMOSPHERE OF HYDROGEN		IN ATMOSPHERE OF CARBON DIOXIDE	
	Ascorbic acid per 20 ml. broth	Loss	Ascorbic acid per 20 ml. broth	Loss
	mgm.	*per cent*	*mgm.*	*per cent*
Control (start)..............	0.74		0.72	
Control (5 hours)...........	0.13	82	0.40	44
A. aerogenes (A101)..........	0.24	68	0.47	35
E. coli (Crooks).............	0.32	57	0.42	42
E. coli (A1)................	0.43	42	0.52	28
E. coli (A2)................	0.30	59	0.53	26
E. coli (A3)................	0.34	54	0.55	24
E. coli (A4)................	0.32	57	0.60	17
E. coli (A11)...............	0.36	51	0.50	30
E. coli (A12)...............	0.31	58	0.55	24
E. coli (A20)...............	0.33	55	0.50	30
E. coli (A21)...............	0.35	53	0.50	30
E. coli (A25)...............	0.39	47		
E. coli (A30)...............	0.41	45		
S. pullorum.................	0.18	76		
S. aertrycke................	0.33	55	0.40	44
S. enteritidis..............	0.32	57	0.60	17
E. typhosa.................	0.29	61		
P. vulgaris.................	0.19	74	0.50	30
B. subtilis......	0.16	78	0.40	44

the oxidation of ascorbic acid, the evidence does not prove that the carbon dioxide is the only inhibiting factor involved.

In order to determine the rôle, if any, of carbon dioxide production by bacteria in the prevention of the oxidation of ascorbic

TABLE 6

The total amounts of dissolved gas and of carbon dioxide produced by bacteria in glucose broth in five hours at 37°C. and the oxidation of ascorbic acid under the same conditions

ORGANISM	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS	TOTAL VOL. OF GAS AS CC. PER 100 ML. OF BROTH	VOL. OF CARBON DIOXIDE PER 100 ML. OF BROTH
	mgm.	*per cent*		
Control (start)...............	0.75			
Control (5 hours)...........	0.10	86	2.1	0.0
A. aerogenes (A101).........	0.46	39	12.8	4.7
E. coli (Crooks).............	0.52	30	6.2	1.9
E. coli (A1).................	0.55	26	8.8	3.4
E. coli (A2).................	0.39	48	7.7	2.2
E. coli (A3).................	0.45	37	9.0	1.9
E. coli (A4).................	0.41	43	8.0	1.9
E. coli (A11)................	0.47	35	9.6	2.3
E. coli (A12)................	0.45	37	9.3	3.2
E. coli (A20)................	0.49	29	8.8	2.1
E. coli (A21)................	0.47	35	9.2	2.8
E. coli (A25)................	0.43	42	8.8	2.3
E. coli (A30)................	0.47	35	8.3	2.1
S. pullorum..................	0.12	84	2.5	0.0
S. aertrycke.................	0.37	50	8.2	1.8
S. enteritidis...............	0.40	46	9.3	1.9
E. typhosa..................	0.20	73	3.2	0.0
P. vulgaris..................	0.19	75	2.4	0.2
B. subtilis..................	0.11	85	2.0	0.0

TABLE 7

The influence of dissolved carbon dioxide on the rate of oxidation of ascorbic acid in uninoculated glucose broth in five hours at 37°C.

GROUP	DISSOLVED CARBON DIOXIDE PER 100 ML. BROTH	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS
	cc.	*mgm.*	*per cent*
Control (start).................	0.0	0.77	
Control (5 hours)..............	0.0	0.08	90
Series A, plus CO_2.............	2.0	0.19	75
Series B, plus CO_2.............	4.6	0.30	61
Series C, plus CO_2.............	13.4	0.30	61

acid, an experiment similar to the last one was conducted, only in this case three series of an uninoculated medium containing

graduated amounts of dissolved carbon dioxide were used. Ascorbic acid was added to each flask and carbon dioxide dissolved in the medium by placing the flasks in anaerobe jars and adding carbon dioxide to the atmosphere of the jars. When the medium contained the desired amount of this gas (previously determined) the anaerobe jars containing the flasks of medium were incubated for five hours at 37°C. At the end of this period the ascorbic acid and carbon dioxide contents of each series were determined. The data presented in table 7 show that the presence of carbon dioxide in glucose broth did exert a small inhibitory effect on the rate of oxidation of ascorbic acid. However, this inhibitory effect was not as marked as that produced by bacteria which evolve carbon dioxide when grown in glucose broth. The uninoculated glucose broth used in the last experiment contained a greater concentration of dissolved carbon dioxide than was produced by bacteria under similar conditions. This fact indicated that carbon dioxide production by bacteria is not the only factor in the mechanism whereby they inhibit the oxidation of ascorbic acid.

Assuming that the carbon dioxide production by bacteria was one factor influencing their inhibitory action on the oxidation of ascorbic acid, another set of experiments was conducted in order to find out whether bacteria exert this effect by combinations of three factors, namely: (1) carbon dioxide production, (2) removal of oxygen from the medium, and (3) the formation of un-ionized copper complexes. In this experiment uninoculated glucose broth was used as the medium, and the flasks were incubated in anaerobe jars for five hours at 37°C. The influence of carbon dioxide was determined by adding the gas at 20 pounds pressure to the atmosphere in the anaerobe jars. The effect of the removal of oxygen from the medium by bacterial action was simulated by evacuating the anaerobe jars with a vacuum pump; and the formation of un-ionized copper complexes by the bacteria was duplicated by adding 0.03 gram of 8-hydroxyquinoline to each 100 ml. of medium. (It has been previously shown by Barron, Barron, and Klemperer (1936) that 8-hydroxyquinoline destroys

the catalytic action of small amounts of copper on the oxidation of ascorbic acid.)   The results are presented in table 8.

Each of the above factors exerted a certain amount of inhibitory action on the oxidation of ascorbic acid, but when they were used in combinations of two together, or of all three together, their combined inhibitory effect was greater than the total inhibitory effect of these three factors, namely, carbon dioxide, vacuum, and 8-hydroxyquinoline, employed separately. In

TABLE 8

The inhibitory effect of carbon dioxide, vacuum, and 8-hydroxyquinoline on the oxidation of ascorbic acid in uninoculated glucose broth in 5 hours at 37°C.

EXPERIMENTAL GROUP	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS	INHIBITION OF ASCORBIC ACID OXIDATION*
	mgm.	*per cent*	*per cent*
Control (start).........................	0.79		
Control (5 hours).......................	0.10	87	
Control plus 8-hydroxyquinoline.........	0.12	85	2
Control plus vacuum....................	0.19	76	11
Control plus carbon dioxide.............	0.24	70	17
Control plus 8-hydroxyquinoline plus vacuum..............................	0.32	60	27
Control plus 8-hydroxyquinoline plus carbon dioxide........................	0.47	40	47
Control plus vacuum plus carbon dioxide	0.47	40	47
Control plus vacuum plus carbon dioxide plus 8-hydroxyquinoline...............	0.69	13	74

* Note: The per cent inhibition of ascorbic acid oxidation refers to the difference between the per cent loss of ascorbic acid in the control medium and that in the control media plus the substance under consideration.

comparing these data with those previously obtained with bacteria it would seem that the ability of the various bacteria to inhibit the oxidation of ascorbic acid might be due to their ability to form un-ionized copper complexes, and to their production of carbon dioxide in, and their removal of oxygen from, the medium. It is not unreasonable to suspect that the intensity of the action of the above three mechanisms would vary with different species of bacteria and with their stage of growth. When studied separately the inhibitory action of carbon dioxide was greater

than that of either a vacuum or of 8-hydroxyquinoline. The 8-hydroxyquinoline was the least active in this respect, but when this substance was used in combination with carbon dioxide or with a vacuum the resultant inhibitory action was more pronounced.

If it is assumed that all bacteria in their growth in culture media are capable of forming un-ionized copper complexes, then bacterial species which produce carbon dioxide in their metabolic processes should exert a more marked inhibitory effect on the oxidation of ascorbic acid than those organisms which do not produce carbon dioxide and, also, the inhibitory effect of carbon dioxide-producing species should be greater than that of carbon dioxide alone when added to uninoculated culture media. Likewise, those species producing little or no carbon dioxide should exert a stronger inhibitory action when grown in an atmosphere of this gas. Also, species such as *Escherichia coli*, when they are grown in nutrient broth to which carbon dioxide is added, should exert an inhibitory effect similar to that which they exert when grown in glucose broth. The reduced oxygen tension of the medium in which the bacteria are grown, which was duplicated by the use of a vacuum, is another factor which should influence the ability of bacteria to inhibit the oxidation of ascorbic acid. However, this factor is likely to be variable due to the cultural conditions and the changing vigor of the bacterial growth.

If the data in tables 1, 5, 6, and 8 are compared it will be seen that the observations made in this investigation substantiate the above explanation of the mechanism by which bacteria inhibit the oxidation of ascorbic acid.

The influence of killed cultures of bacteria and of cell-free culture filtrates on the oxidation of ascorbic acid

It has been suggested that the inhibitory action of bacteria on the oxidation of ascorbic acid is due principally to the production of carbon dioxide and the reduced oxygen tension of the medium as a result of their metabolic activities. If such is the case it would seem that killed cultures of the bacteria, or the cell-free filtrates from such cultures, would not exhibit the inhibitory

action observed with actively growing cultures of bacteria. Three series of tests were conducted to investigate this hypothesis.

Twenty-four-hour cultures of *Escherichia coli* and of *Salmonella aertrycke* in glucose broth were filtered through Berkefeld filters. Ascorbic acid was added to these culture filtrates and they were incubated. At the end of five hours the per cent of loss of ascorbic acid was determined. Also, twenty-four-hour cultures of *Aerobacter aerogenes*, *Escherichia coli*, *Proteus vulgaris*, and *Bacillus subtilis* were killed by 15 pounds steam pressure for 15 minutes, and by the addition of 4.0 ml. of 20 per cent phenol per culture, respectively. Ascorbic acid was added to the killed cultures, and at the end of a five-hour incubation period at 37°C. the per cent of loss of ascorbic acid was determined. In all cases it was found that the absence of actively growing bacteria, whether they were removed from the medium by filtration or were killed, resulted in the loss of the ability of the cultures to inhibit the oxidation of ascorbic acid significantly. Thus, further evidence is provided to show that in order to prevent the oxidation of ascorbic acid in culture media actively growing bacteria must be present.

The ability of bacteria to reduce dehydroascorbic acid

The only report which has been found in the literature pertaining to the reduction of dehydroascorbic acid by bacteria is that of Tkachenko (1936) who reported that such a reduction did take place in cultures of several species of "Lactobacilli." Dehydroascorbic acid was prepared according to the method of Kohman and Sanborn (1937) by oxidizing ascorbic acid in aqueous solution with iodine. Eleven strains of *Escherichia coli*, and one strain each of *Aerobacter aerogenes*, *Salmonella pullorum*, *Salmonella aertrycke*, and *Salmonella enteritidis* were studied with reference to their ability to reduce dehydroascorbic acid. The acid was added to 24-hour cultures of these organisms in glucose broth. At the beginning of the experiment the ascorbic acid in the medium was all present in the dehydro-form, but at the end of five hours two of the strains of *Escherichia coli* had reduced approximately 90 per cent of the dehydroascorbic acid to its

equivalent of ascorbic acid, as may be seen in table 9. Of the organisms studied only these two strains of *Escherichia coli* were capable of bringing about this reduction. Repeated tests furnished definite proof that the two strains of *Escherichia coli* were unique in this respect among the strains employed.

TABLE 9

The reduction of dehydroascorbic acid to ascorbic acid by 24-hour glucose broth cultures of bacteria in five hours at 37°C.

ORGANISM	ASCORBIC ACID PER 20 ML. OF BROTH	REDUCTION OF DEHYDROASCORBIC ACID TO ASCORBIC ACID
	mgm.	*per cent*
Control (start)...........................	0.62*	
Control (5 hours)........................	0.00	0
A. aerogenes (A101)......................	0.04	6
E. coli (Crooks).........................	0.54	87
E. coli (A1).............................	0.58	94
E. coli (A2).............................	0.01	2
E. coli (A3).............................	0.01	2
E. coli (A4).............................	0.01	2
E. coli (A11)............................	0.01	2
E. coli (A12)............................	0.01	2
E. coli (A20)............................	0.01	2
E. coli (A21)............................	0.01	2
E. coli (A25)............................	0.01	2
E. coli (A30)............................	0.01	2
S. pullorum.............................	0.01	2
S. aertrycke............................	0.01	2
S. enteritidis...........................	0.01	2

* Ascorbic acid equivalent of dehydroascorbic acid.

SUMMARY

1. It has been found that certain bacteria, particularly members of the coliform group, inhibited the oxidation of ascorbic acid in culture media. The stronger inhibitory action was observed with the more actively growing cultures in which the largest numbers of bacteria were present.

2. Ascorbic acid was rapidly oxidized in uninoculated nutrient broth at 37°C., and this oxidation was retarded by the presence of carbon dioxide, by 8-hydroxyquinoline, and in the absence of oxygen.

3. It is suggested that growing bacteria inhibit the oxidation of ascorbic acid by certain combinations of three factors, namely: (1) the formation of un-ionized copper complexes whereby the catalytic action of the copper is destroyed, (2) the production of carbon dioxide with the subsequent saturation of the medium with it, and (3) the lowering of the oxygen tension of the medium. The most effective inhibitory action was obtained by a combination of all three of these factors together.

4. Two strains only of *Escherichia coli* were able to reduce dehydroascorbic acid to ascorbic acid, while the other bacteria studied did not appear to possess this ability.

5. The inhibitory action of bacteria on the oxidation of ascorbic acid was not observed in relatively large concentrations of this substance because the acidity of the medium was lowered below the point where effective bacterial growth could take place.

6. Killed bacterial cultures, or cell-free filtrates from living bacterial cultures, did not possess the inhibitory action on the oxidation of ascorbic acid that was observed with actively growing bacterial cultures.

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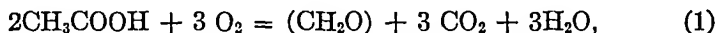
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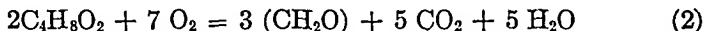
C. E. CLIFTON AND W. A. LOGAN

Department of Bacteriology and Experimental Pathology, Stanford University, California

Until recently, it has been generally assumed that the conversion under highly aerobic conditions of relatively simple, non-nitrogenous compounds by washed suspensions of bacteria is limited to an oxidation of the substrate to carbon dioxide and water. Barker (1936), Geisberger (1936) and Clifton (1937) have shown that the oxidation does not proceed to completion, but that instead, a portion of the substrate is apparently assimilated by the cells. For example, the oxidative assimilation of acetate and of butyrate by *Pseudomonas calco-acetica* may be represented as

$$2CH_3COOH + 3\ O_2 = (CH_2O) + 3\ CO_2 + 3H_2O, \qquad (1)$$

and

$$2C_4H_8O_2 + 7\ O_2 = 3\ (CH_2O) + 5\ CO_2 + 5\ H_2O \qquad (2)$$

These equations indicate that approximately three-fourths of the oxygen required for the complete combustion of these substrates is consumed, with the production of carbon dioxide, water and a substance, or substances, having the empirical composition of a carbohydrate.

The oxidative assimilation of butyrate is of particular interest since the respiratory quotient (R.Q.) during the stage of rapid oxidation is approximately 0.68 while the theoretical R.Q. for complete combustion is 0.8. The rate of oxygen consumption abruptly decreases to a level near that of the control by the

time that three-fourths of the oxygen required for complete combustion has been consumed and the R.Q. increases to 0.94, a value characteristic of the respiration of the control suspension. However, in the presence of suitable concentrations of sodium azide (NaN_3) or of 2:4 dinitrophenol (α DNP) the oxidation proceeds to completion at a quite constant rate and the R.Q. throughout is equal to the theoretical value, 0.8.

The oxidative assimilation of lactic acid in washed suspensions and in cultures of *Escherichia coli* has recently been described by Clifton and Logan (1938). The oxygen uptake in a M/15 phosphate buffer of pH 7.1, proceeds at a rapid and quite constant rate until approximately two-thirds of the oxygen required for complete combustion has been consumed. Then the rate of oxygen consumption abruptly decreases and approaches the level of the substrate-free control, a behavior first noted by Cook and Stephenson (1928). These results, together with an observed R.Q. of 1.0, suggest that the oxidation of lactate is an oxidative assimilation process which may be represented as

$$CH_3CHOHCOOH + 2\,O_2 = (CH_2O) + 2CO_2 + 2\,H_2O \qquad (3)$$
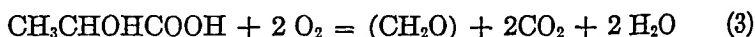
In a synthetic medium containing lactate as the only source of carbon the rate of oxygen consumption also breaks abruptly when approximately two-thirds of the theoretical amount for complete combustion has been consumed, thus suggesting that equation 3 also represents the primary phase of assimilation in cultures of *E. coli*. Furthermore, in buffered suspensions of *E. coli* the oxidation of lactate proceeds to completion in the presence of critical concentrations of NaN_3 or of α DNP. The addition of these agents to the culture medium proves to be inhibitory to growth.

The studies to be reported here deal primarily with the oxidative assimilation of acetate, pyruvate, glycerol, fumarate, succinate and glucose by washed suspensions of *E. coli* and of certain of these substrates in cultures of the same organism.

EXPERIMENTAL

The strain of *Escherichia coli* (K-12) employed in these studies was grown on nutrient agar at 30°C.; after 20 hours the growth

was washed off with distilled water and centrifuged. The organisms were washed twice by centrifugation and finally suspended in distilled water. This stock suspension showed no marked decrease in metabolic activity on standing in the refrigerator for several days. It was diluted as needed with phosphate buffer to a final concentration of m/15 and a pH of 7.1 unless otherwise stated. Stock solutions of the various substrates were carefully prepared, neutralized to pH 7.1 if necessary and sterilized by filtration through Jena sintered glass filters ("G 5 on 3").

A synthetic medium (Clifton, Cahen and Morrow, 1936) containing ammonium chloride as the source of nitrogen and one organic compound as the source of carbon was employed in the studies on the relation between respiration and assimilation in actively proliferating cultures of *E. coli*. In all studies reported the synthetic medium was rather heavily inoculated with a 20-hour peptone culture. Control tests indicated that the inoculum did not contain an appreciable quantity of oxidizable material. The rates and extent of oxygen consumption and of carbon dioxide production at 30°C. were determined by the usual Warburg technic, a total volume of 2.2 ml. of liquid being employed in flasks of 16–17 ml. capacity shaken at a rate of 100 cycles per minute. NaN_3 or α DNP was always added to the bacterial suspension before the flasks were placed in the manometers. The substrate was tipped in from a side-arm of the flask at zero time, 15–20 minutes after the flasks were placed in the water bath. No marked differences in the results were observed when both poison and substrate were added to the bacterial suspension at zero time, the above procedure being adopted primarily for convenience.

The oxidative assimilation of acetate

The oxidation of acetate by washed suspensions of *E. coli* proceeds over a pH range of 5.6 to 7.7 in a manner similar to that previously reported for *Pseudomonas calco-acetica* (Clifton, 1937) and for *E. coli* (Cook and Stephenson, 1928). The rate of oxygen consumption remains at a relatively constant level,

irrespective of the initial concentration of acetate, over the range studied, M/50–M/400, until approximately three-fourths of the oxygen required for complete combustion has been consumed. At this time the rate of oxygen consumption decreases abruptly and approaches a level near that of the control suspension to which no acetate had been added. The R.Q. in phosphate buffer was approximately 1.0. No correction was applied for the blank respiration since it appears to be negligible in the presence of a readily utilizable substrate. If a correction for the blank respiration were applied, the oxygen consumed during the oxidation of the acetate would amount to somewhat less than the reported value of three-fourths of that required for complete combustion.

Krebs (1937) has reported that the oxidation of acetate by *E. coli* in a saline-phosphate buffer of pH 6.5 goes to completion at a quite constant rate. Very little free carbon dioxide is formed in a saline-bicarbonate suspension in equilibrium with a 95 per cent oxygen-5 per cent carbon dioxide gas mixture during the initial stage of oxidation, while in the latter stages the ratio of carbon dioxide liberated to oxygen consumed is nearly 1.0. This observation suggested that the oxidation of acetate proceeds by stages. In the first stage only bicarbonate is set free, i.e., not more than one molecule of CO_2 is formed per molecule of acetate oxidized, while in the second stage carbon dioxide is set free.

We were unable to confirm these observations of Krebs, the oxidation of acetate by our strain of *E. coli* in Krebs saline-phosphate buffer mixtures of pH 6.5 to 7.5 going only about three-fourths to completion, even when the air in the Warburg flasks was replaced by oxygen. Also, no appreciable lag in carbon dioxide production was observed in the saline-bicarbonate buffer in equilibrium with a 95 per cent oxygen-5 per cent carbon dioxide gas mixture and the rate of carbon dioxide liberation was always approximately equal to the rate of bicarbonate formation in the studies on the kinetics of the oxidation of acetate.

Since the amount of acetate added to the bacterial suspension

at zero time was known and the amounts of oxygen consumed and of carbon dioxide produced were determined, it is possible to deduce the empirical composition of the other product, or products, just as the synthesis of a carbohydrate is deduced from the value of the photosynthetic quotient. Applying this mode of reasoning to the experimental data for the oxidation of acetate it is apparent that the balanced equation for this reaction may be represented as

$$2 \, CH_3COOH + 3 \, O_2 = (CH_2O) + 3 \, CO_2 + 3 \, H_2O \qquad (4)$$
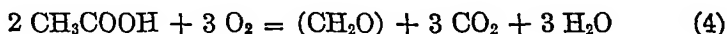
That is, the acetic acid is oxidized to form a carbohydrate, carbon dioxide and water. It is true that no direct evidence is available that a carbohydrate is actually formed and stored by *E. coli* but Geisberger (1936) has demonstrated an increase in the volutin content of *Spirillum serpens* in similar experiments with calcium lactate as the substrate. Also the fact that the oxidation of lactate in either buffered suspensions or in actively proliferating cultures proceeds only two-thirds to completion lends indirect evidence for the hypothesis of oxidative assimilation by actively respiring but non-proliferating cells. Furthermore, possible explanations for the oxidation stopping short of completion have been shown to be untenable (Clifton, 1937). Only formate, of all the compounds studied, is oxidized completely to carbon dioxide and water by *E. coli*.

The oxidation of acetate in phosphate buffer or saline-bicarbonate proceeds to completion in the presence of M/400 NaN_3 or of M/2000 α DNP in accordance with the equation

$$CH_3COOH + 2 \, O_2 = 2 \, CO_2 + 2 \, H_2O \qquad (5)$$

NaN_3 or α DNP in the concentrations employed had no appreciable effect on the rate of oxygen consumption by the control suspension. Also the rate of oxygen consumption in the presence of concentrations of NaN_3 or α DNP just sufficient to prevent assimilation is not markedly different from the rate observed in the absence of the poisons. Still higher concentrations of these agents do, however, inhibit respiration.

The oxidation of three-carbon compounds

The oxidation of propionic acid proceeds in a manner similar to that described above for acetate. However, propionate is not as readily oxidized as acetate and the oxidative assimilation of this compound was not studied in detail. The oxidation of the hydroxy-propionic acid (lactic) has been described (Clifton and Logan, 1938) and appears to be an oxidative assimilation proceeding according to equation 3, two-thirds of the amount of oxygen (4 atoms per mol of lactate) required for complete combustion being consumed. The oxidation of the keto-propionic acid (pyruvic) proceeds in a manner similar to that described for lactate, with the exception that the amount of oxygen consumed amounts to three-fifths of that required for complete combustion. The oxygen consumed during the oxidation of pyruvate corresponds to three atoms of oxygen per molecule, thus agreeing with the observation of Stephenson (1928) that lactate passes through the stage of pyruvate during aerobic oxidation by loss of two atoms of hydrogen.

Pyruvate is somewhat unsatisfactory for quantitative studies since it is unstable in solution. The following data are therefore presented as tentative only. The R.Q. in a limited number of tests was 1.6 or higher during the first stages of oxidation and decreased to 1.36 to 1.38 by the time the oxidation reached three-fifths of completion. These results suggest that the oxidative assimilation of pyruvate may be approximately represented as

$$2\ CH_3COCOOH + 3\ O_2 = 2\ (CH_2O) + 4\ CO_2 + 2\ H_2O \qquad (6)$$
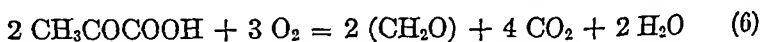
The oxidation of pyruvate by *E. coli* approaches completion in the presence of $M/200$ NaN_3 or $M/2000$ α DNP, as illustrated by typical results presented in figure 1, *A*. The rate of oxygen consumption is inhibited about 50 per cent in the presence of $M/2000$ α DNP while no marked inhibition is observed in the presence of concentrations of α DNP less than $M/4000$. However, the extent to which the oxidation of pyruvate approaches completion decreases with decreasing concentration of α DNP being approximately 85, 75 and 65 per cent, respectively, in $M/4000$, $M/8000$ and $M/16,000$ solutions of α DNP.

The amounts of oxygen consumed during the growth of *E. coli*

in the synthetic medium containing pyruvate as the carbon and energy source were approximately equal to three-fifths of the amounts required for complete oxidation of pyruvate to carbon dioxide and water. Typical time-growth, time-oxygen consumption and time-carbon dioxide production relationships are presented in figure 1, *B*. Values for the oxygen consumption and carbon dioxide production per ml. per hour and per cell per
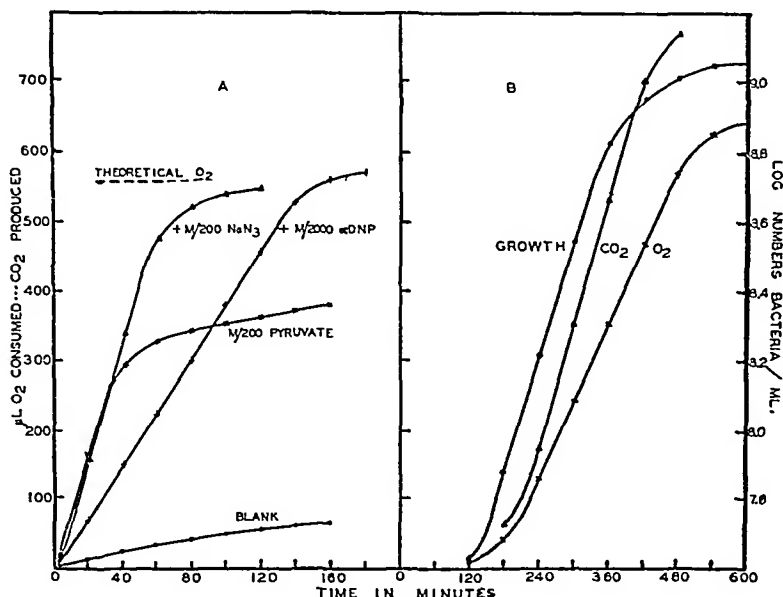

FIG. 1. THE OXIDATION OF PYRUVATE BY E. COLI

(*A*) the influence of NaN₃ and of αDNP on the oxidation of 2 ml. of м/200 pyruvate. (*B*) typical time-growth, time-oxygen consumption and time-carbon dioxide production relationships observed in 2 ml. of a synthetic medium, м/100 with respect to pyruvate.

hour (computed by Buchanan's (1930) formula), together with the observed R.Q.'s are presented in table 1.

The oxidation of glycerol by *E. coli* was studied in considerable detail, as this substrate is very stable under ordinary conditions and can readily be obtained in a pure state. Typical results on the rate and extent of oxidation of different concentrations of glycerol are presented in figure 2. In figure 2, *A*, the total oxygen consumption is plotted against time while in 2, *B*, the

oxygen consumed per ten-minute interval is plotted against the corresponding time. This latter method of plotting the results clearly shows the marked break in the rate of oxygen consumption which is observed by the time the oxidation reaches four-sevenths of completion as shown in figure 2, A. It can readily be seen in this latter figure that the break occurs around four-sevenths of completion irrespective of the initial concentration of glycerol over the range reported. The break is also observed in the same region in higher concentrations of glycerol, over a pH range of 5.6 to 7.7, or at a temperature of 37.5°C.

TABLE 1

Influence of the age of a culture of E. coli on the rate of oxygen consumption and of carbon dioxide production per ml. of a M/100 pyruvate-inorganic medium

| TIME | NUMBER OF BACTERIA AT END OF TIME INTERVAL | MICROLITERS OF O_2 CONSUMED | | MICROLITERS OF CO_2 PRODUCED | | R.Q. |
		per ml. per hour	per cell per hour	per ml. per hour	per cell per hour	
minutes						
0–120	4.2×10^7	5		7		1.40
120–180	7.6×10^7	18	31.3×10^{-8}	28	48.7×10^{-8}	1.55
180–240	19.5×10^7	40	31.5×10^{-8}	55	43.4×10^{-8}	1.38
240–300	36.8×10^7	58	21.1×10^{-8}	88	31.1×10^{-8}	1.52
300–360	66.8×10^7	59	11.7×10^{-8}	90	18.5×10^{-8}	1.52
360–420	91.2×10^7	56	7.1×10^{-8}	75	9.6×10^{-8}	1.34
420–480	105.0×10^7	50	5.3×10^{-8}	48	4.9×10^{-8}	0.96
480–540	113.0×10^7	31	2.7×10^{-8}	29	2.6×10^{-8}	0.93
540–600	116.0×10^7	8	0.7×10^{-8}	9	0.8×10^{-8}	1.12
Total		325		429		1.32

The theoretical R.Q. for the oxidation of glycerol is 0.86—while the R.Q. observed in phosphate buffer was in the neighborhood of 0.75 in the early stages of oxidation and approached a value of 0.80 by the time the oxidation approached four-sevenths of completion. A similar behavior was observed in bicarbonate buffer in equilibrium with a 5 per cent carbon dioxide-95 per cent oxygen gas mixture, the R.Q. up to the break being approximately 0.80. No change (outside of the experimental limits of error) in the concentration of bicarbonate was observed during the oxidation of glycerol, thus ruling out the possibility of carbon dioxide

being bound, or released due to the formation of an acidic by-product of oxidation.

The above results indicate that the oxidative assimilation of glycerol by *E. coli* may be approximately represented as

$$2 \, C_3H_8O_3 + 4 \, O_2 = 3 \, (CH_2O) + 3 \, CO_2 + 5 \, H_2O \qquad (7)$$
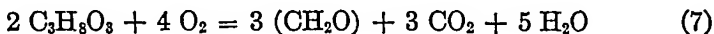
This equation shows that four-sevenths of the amount of oxygen required for complete combustion is consumed with the pro-
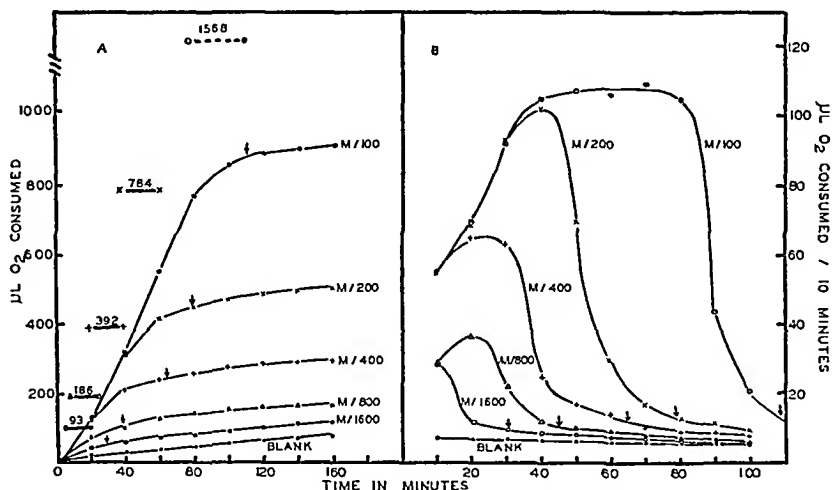

FIG. 2. THE OXIDATION OF 2 ML. OF DIFFERENT CONCENTRATIONS OF GLYCEROL BY E. COLI

Arrows indicate time at which glycerol is assumed to be decomposed, the total oxygen required for complete combustion being indicated by the heavy horizontal lines.

duction of 1.5 mols of a carbohydrate of empirical composition CH_2O per mol of glycerol utilized. The R.Q. of 0.75 postulated by this equation is in fair agreement with the observed R.Q.'s reported above.

The oxidation of glycerol approaches completion in the presence of M/200 NaN_3 or of M/2000 α DNP. Typical results are presented in figure 3, *A*. The observed R.Q.'s are in the neighborhood of the theoretical value, 0.86, and are recorded in table 2.

The amounts of oxygen consumed during the growth of *E. coli*
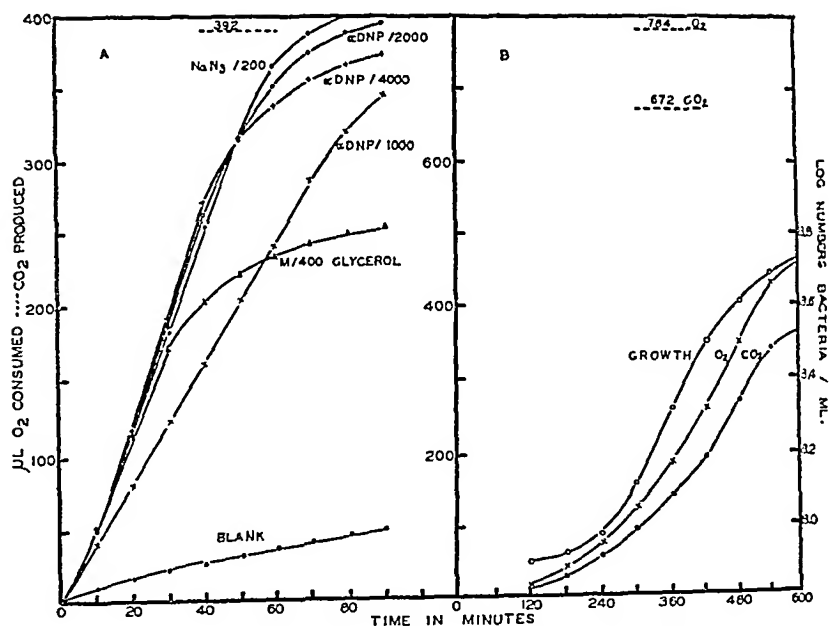
FIG. 3. THE OXIDATION OF GLYCEROL BY E. COLI

(A) the influence of NaN₃ and of αDNP on the oxidation of 2 ml. of M/200 glycerol. (B) typical time-growth, time-oxygen consumption and time-carbon dioxide production relationships observed in 2 ml. of a synthetic medium, M/200 with respect to glycerol.

TABLE 2

Oxygen consumption, carbon dioxide production and R.Q.'s observed during the oxidation of 2 ml. of M/200 glycerol by E. coli

	MINUTES	O_2	CO_2	R.Q.
Glycerol only............	30	225	170	0.75
	60	381	295	0.77
	90	425	358	0.84
	120	463	370	0.80
M/200 NaN₃.............	20	175	136	0.78
	50	481	411	0.86
	70	617	518	0.83
	90	690	578	0.84
M/2000 αDNP...........	30	145	125	0.86
	60	337	274	0.81
	90	551	473	0.86
	120	676	589	0.87

in the synthetic medium containing glycerol as the carbon and energy source were approximately equal to four-sevenths of the amounts required for the complete oxidation of glycerol to carbon dioxide and water, thus again illustrating the similarity between assimilation in non-proliferating suspensions and in cultures of bacteria. Typical time-growth, time oxygen consumption and time-carbon dioxide production relationships observed in a M/200 glycerol medium are presented in figure 3, *B*, and in table 3.

TABLE 3

Influence of the age of a culture of E. coli on the rate of oxygen consumption and of carbon dioxide production in a M/200 glycerol-inorganic medium

| TIME	NUMBER OF BAC-TERIA/ML. AT END OF TIME INTERVAL	MICROLITERS OF O_2 CONSUMED		MICROLITERS OF CO_2 PRODUCED		R.Q.
		per ml. per hour	per cell per hour	per ml. per hour	per cell per hour	
minutes						
0–120	7.9×10^7	5		4		0.80
120–180	8.3×10^7	15	14.7×10^{-8}	12	11.7×10^{-8}	0.80
180–240	8.9×10^7	17	21.5×10^{-8}	13	16.4×10^{-8}	0.76
240–300	13.0×10^7	24	22.2×10^{-8}	17	15.7×10^{-8}	0.71
300–360	20.5×10^7	28	16.7×10^{-8}	21	12.6×10^{-8}	0.75
360–420	30.2×10^7	36	14.5×10^{-8}	28	11.3×10^{-8}	0.75
420–480	40.3×10^7	49	13.9×10^{-8}	39	11.1×10^{-8}	0.79
480–540	48.8×10^7	44	9.9×10^{-8}	37	8.3×10^{-8}	0.84
540–600	52.5×10^7	14	2.8×10^{-8}	12	2.4×10^{-8}	0.86
Total		232		183		0.79

The oxidation of fumarate and succinate

The oxidation of fumarate by *E. coli* tends to go approximately two-thirds to completion before the rate of oxygen consumption falls to a level near that of the control without added substrate. Since the observed R.Q. was approximately 1.50 (theoretical 1.33) the oxidative assimilation of fumarate may be represented as

$$C_4H_4O_4 + 2 O_2 = (CH_2O) + 3 CO_2 + H_2O \tag{8}$$
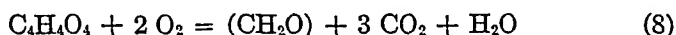
During the oxidation of succinate by *E. coli* one atom more of oxygen is consumed than during the oxidation of fumarate. This difference in oxygen consumption is in agreement with the

observations of Quastel and Wheatley (1931) that the normal course of biological oxidation of succinate proceeds through fumarate. The observed oxygen consumption of approximately five-sevenths of the theoretical and an R.Q. of 1.20 (theoretical 1.14) suggests that the oxidative assimilation of succinate may be represented as

$$C_4H_6O_2 + 2\ 1/2\ O_2 = (CH_2O) + 3\ CO_2 + H_2O \tag{9}$$
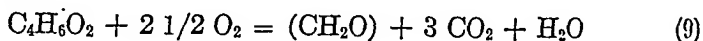
The oxidation of fumarate, succinate and related compounds will be reported in detail at a later date. In a limited number of experiments the presence of NaN_3 or of α DNP appears to inhibit the oxidation of fumarate and succinate to a much greater extent than that of the other substrates reported in this paper. For example, the oxidation of succinate by *E. coli* was inhibited almost 50 per cent by M/16,000 α DNP, although the oxidation did tend to approach completion under these conditions, while the oxygen consumption in the presence of M/2000 α DNP was only approximately equal to that of the control suspension. This marked inhibitory action of NaN_3 or of α DNP on the oxidation of fumarate and succinate suggests that the fumarate-succinate system does not play an important rôle as a respiratory catalyst (hydrogen carrier) in the oxidation of acetate, propionate, lactate, pyruvate or glycerol by *E. coli* under aerobic conditions. However, the possibility remains that the fumarate-succinate system may be involved in the assimilatory process.

The oxidation of glucose

The oxidation of glucose by suspensions of *E. coli* goes approximately one-half to completion, in agreement with the observation of Cook and Stephenson (1928). Krebs (1937) has reported that the oxidation of glucose by *E. coli* goes 70 per cent to completion at 40°C. and a pH of 6.8. However, inspection of his data indicates that a marked break in oxygen consumption occurs in the neighborhood of 50 per cent of completion. Since the R.Q. observed during the oxidation of glucose is 1.0, the assimilatory process may be represented as

$$C_6H_{12}O_6 + 3\ O_2 = 3\ (CH_2O) + 3\ CO_2 + 3\ H_2O \tag{10}$$
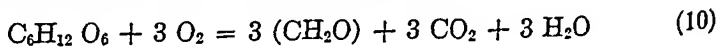
The oxidation of glucose tends to approach completion in the presence of M/400 NaN$_3$ or M/2000 α DNP.   In a typical experiment 664 μl. of oxygen were consumed and 659 μl. of carbon dioxide were produced in 160 minutes in the presence of M/2000 α DNP while in the same time the gaseous exchange in the absence of this agent was only 439 μl. of oxygen and 429 μl. of carbon dioxide.   The oxygen consumption and carbon dioxide production in the latter case had reached a level near that of the control when approximately 340 μl. of oxygen had been consumed.

TABLE 4

Influence of the age of a culture of E. coli on the rate of oxygen consumption in a M/200 glucose-inorganic medium

TIME	NUMBER OF BACTERIA/ ML. AT END OF TIME INTERVAL	MICRÔLITERS OF O$_2$ CONSUMED	
		per ml. per hour	per cell per hour
minutes			
0–120	6.5×10^7	10	
120–180	13.6×10^7	21	21.7×10^{-8}
180–240	32.3×10^7	34	15.7×10^{-8}
240–300	63.1×10^7	53	11.4×10^{-8}
300–360	92.9×10^7	80	10.4×10^{-8}
360–420	106.7×10^7	41	4.1×10^{-8}
420–480	114.3×10^7	11	1.0×10^{-8}
480–540	117.5×10^7	4	0.3×10^{-8}
Total		254	.

A marked break in the rate of oxygen consumption during the growth of *E. coli* in the synthetic medium plus glucose was noted by the time that the oxidation had proceeded to between thirty-five to forty per cent of completion.   Typical results of oxygen consumption and of growth in the glucose medium are recorded in table 4, carbon dioxide values being omitted as they are identical with the values reported for oxygen within the limits of experimental error.   Glucose could no longer be detected in the medium immediately after the marked break in oxygen consumption was observed.

Since the concentration of phosphates was higher in the sus-

pensions than in the cultures, it was deemed advisable to repeat the studies with glucose as the source of energy and of carbon for growth. Accordingly three parts of the inorganic medium were diluted with one part of M/7.5 phosphate buffer of pH 7.1. The rate of oxygen consumption in this phosphate rich medium was approximately equal to that observed in the normal medium, but the marked break in oxygen consumption was observed somewhat nearer 50 per cent of completion. This effect remains unexplained, but preliminary tests show that it is not due to a change in pH of the medium. No appreciable difference was observed in the extent of oxidation of pyruvate or of glycerol in the normal and in the phosphate-rich medium.

DISCUSSION

The data presented in this paper indicate that the oxidation of a number of organic compounds by suspensions of *E. coli* is not carried to completion, but that instead a portion of the compound is assimilated by the cells, probably in the form of a carbohydrate. Barker (1936), and Geisberger (1936), have reached similar conclusions in regard to the oxidation of a variety of compounds by the colorless alga, *Prototheca zopfii* and various species of the genus *Spirillum*, respectively, while Benoy and Elliott (1937) have reported the synthesis of carbohydrate from a number of organic compounds by rat tissues under conditions similar to those prevailing in manometric experiments. Brück (1933) has demonstrated that iodoacetate inhibits the formation of glycogen from glucose or ethyl alcohol by yeast. This poison has also been shown to inhibit oxidative assimilation by *Pseudomonas calco-acetica* and *Escherichia coli* (Clifton, 1937). A number of other reports in the literature also demonstrate that the oxidation of various substrates is not carried to completion by suspensions of bacteria or by tissue slices. Thus, it becomes apparent that the study of respiration by the manometric technic does not necessarily separate assimilatory and dissimilatory processes.

Winzler (1938) has reported that the heat production during the oxidation of glucose and of acetate by yeast amounts to only 26.65 and 58.7 per cent respectively, of the theoretical

amounts for complete combustion to carbon dioxide and water while the heat production during the fermentation of glucose is 70.5 per cent of the theoretical. Winzler also reached the conclusion that carbohydrate was assimilated during respiration and calculated that the free energy efficiency of this assimilatory process was only 2.9 and 12.2 per cent, respectively, during the oxidation of glucose and of acetate. He also demonstrated that the oxidation of acetate proceeds to completion in the presence of suitable concentrations of α dinitrophenol.

The experiments reported in this paper leave no doubt that it is possible to poison the cells selectively in such a way that synthetical processes are prevented while at the same time respiration proceeds with complete oxidation of the substrate to carbon dioxide and water. This has been proved by the fact that with suitable concentrations of sodium azide or of α dinitrophenol the oxygen consumption and carbon dioxide production approach the theoretical values for complete oxidation before a marked break in the rate of respiration is observed.

Regardless of the initial concentration or total quantity of substrate, the oxygen consumed before the marked break in rate of consumption is observed is always a constant proportion of the total amount required for the oxidation of a given substrate. Therefore, the ratio of synthesis to oxidation must be constant for a given system. This suggests that there is a close connection between dissimilation and assimilation, possibly of the nature of coupled reactions for, as pointed out by Borsook (1935) (see also Clark, 1938), in order for synthesis to occur, i.e., for work to be done by a spontaneous reaction, there must be a simultaneous reaction involving an increase in free energy, and these two processes must be coupled in such a manner that electrons of hydrogen freed in one reaction are transferred to the other. Borsook also pointed out that the reduction of pyruvate to lactate (synthesis of lactate) by *Escherichia coli* in the presence of formate can be blocked by the addition of toluol, but that the reaction can proceed provided an appropriate artificial "carrier" between the two enzyme systems is added.

The inefficient utilization of energy by bacteria and other tissues may be explained as due to the stoichiometric relationship between the free-energy-yielding and the free-energy-requiring coupled reactions, the efficiency of synthesis being dependent upon the relative values of the free energies of the two halves of the reaction. Since the free energies of the assimilatory processes considered are much smaller than the free energies of the oxidations to carbon dioxide and water—that is the substrates are nearer in chemical potential to the cell material than to their oxidation products—there is necessarily much loss of energy. This is demonstrated by the fact that the same amount of assimilation occurs during the oxidation of lactate and pyruvate, or of succinate and fumarate, although the free energy of lactate or of succinate is greater than that of pyruvate or fumarate, respectively. This suggests that assimilation may involve the intermediate products of oxidation rather than the initial substrate.

It is true that the manner of action of NaN_3 or of α DNP in blocking the assimilatory process is still obscure. Experiments with methylene blue as the hydrogen acceptor indicate that NaN_3 does not inhibit the dehydrogenase systems of *E. coli*. Green, Needham and Dewane (1937) are of the opinion that synthesis of carbohydrate from lactate in muscle takes place in the following manner: a trace of phosphoglycerate will react with lactate to produce triosephosphate and pyruvate; this reaction will proceed if the triosephosphate is continually removed by condensation to hexosediphosphate and then to glycogen and phosphate. Given an energy source, the pyruvate formed in the oxido-reduction reaction could be phosphorylated and its conversion into a further supply of phosphoglycerate to react with lactate would follow.

The principle of carbohydrate formation by *E. coli* could possibly be a dehydrogenation of a portion of the substrate with a transfer of this hydrogen by means of a carrier such as coenzyme to phosphoglycerate, the reduced form of the latter being removed by condensation and complex carbohydrate formation. The formation and phosphorylation of pyruvate or related compounds

during the oxidation of the substrate would serve as a continual source of phosphoglycerate to enable the reaction to continue, energy being supplied by coupled reactions during which a portion of the substrate is oxidized to carbon dioxide and water. It has been demonstrated that iodoacetate, an agent shown to block assimilation by *Pseudomonas calco-acetica* and *Escherichia coli*, will block the conversion of triosephosphate into phosphoglycerate. Possibly NaN_3 and α DNP may also exert an inhibitory action in the chain of reactions leading to stored carbohydrate, thus allowing the oxidation to proceed to completion. Studies are in progress to determine if assimilation, and the blocking of assimilation does proceed as postulated above.

It has been demonstrated that the oxidative assimilation of lactate, pyruvate, glycerol or of glucose as observed in buffered suspensions of *E. coli* appears to be the primary assimilatory processes in actively proliferating cultures of this organism. We may therefore postulate that the general assimilatory process may be represented as:

Substrate $+ O_2$ yielding energy $=$ assimilated material $+ CO_2 + H_2O$

much as

$$CO_2 + H_2O + \text{radiant energy} = (CH_2O) + O_2$$
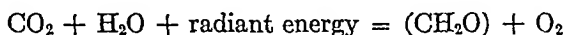
represents the assimilatory process in the green plant.

SUMMARY

It has been shown that the oxidation of acetate, propionate, lactate, glycerol, fumarate, succinate and glucose by washed suspensions of *Escherichia coli* in phosphate or bicarbonate buffers is not carried to completion. The results suggest that a portion of the substrate is assimilated as carbohydrate by the cells, the amount assimilated being dependent on the nature of the substrate and independent of its concentration.

Indirect evidence of this assimilation is presented in studies on the growth of *Escherichia coli* in a synthetic medium, the same relation between the extent of oxidation and assimilation being observed in actively proliferating cultures as in washed suspensions of this organism.

The respiratory and assimilatory processes appear to be closely connected although it is possible to block the assimilatory process and bring about a complete oxidation of the substrate by adding suitable concentration of sodium azide or of α dinitrophenol to the bacterial suspension.

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THE EXTRACELLULAR PROTEOLYTIC SYSTEM OF CLOSTRIDIUM PARABOTULINUM

S. S. ELBERG AND K. F. MEYER

George Williams Hooper Foundation for Medical Research and Department of Bacteriology, University of California, Medical School, San Francisco, California

The enzyme complements of the proteolytic anaerobic bacteria have been widely investigated, but strangely enough *Clostridium botulinum* has been neglected in this respect. In view of the importance of this organism both theoretically and practically, as a producer of a potent neurotoxin, we have attempted to determine the nature and properties of the various enzymes which catalyze proteolysis by this organism and the relation of these enzymes to toxin production.

Wagner, Meyer and Dozier (1925) stated that the gelatinase which was produced in cultures of *C. botulinum* was influenced by the carbohydrate concentration of the medium and they observed a more rapid disappearance of the gelatinase in the presence of carbohydrate than in its absence.

Snipe and Sommer (1928) in their work on purification of botulinus toxin found that a gelatinase, a peptidase, and a lipase were precipitated with the toxin by acidification of the supernatant liquid from cultures.

Maschmann (1927, 1938) has also made a study of the proteolytic enzymes of *C. botulinum*. He found that "there is a proteinase, an aminopolypeptidase and a dipeptidase secreted in the medium during growth." These enzymes were determined by the amino-group titration procedure. They were activated by $M/250$ cysteine, $M/100$ hydrogen cyanide and were unaffected by iodoacetic acid. Maschmann also found that cysteine was necessary before the proteinase would attack clupeine.

The objections, however, to using amino group or carboxyl group determinations for estimating proteinase activity have been clearly stated by Northrop (1932) and Anson (1932). "The splitting of peptide linkages which is what is measured by determination of the number of amino and carboxyl groups freed is due not only to proteinases but also to peptidases which cannot digest proteins. The estimation of the proteinases of anaerobes by Maschmann are thus estimations not of proteinase alone but of proteinases plus peptidases." (Anson, 1938).

EXPERIMENTAL

Northrop (1932) determined that the first effect of proteinases such as pepsin and trypsin on proteins (gelatin, casein and edestin) was to cause a rapid decrease in the viscosity of the protein. A measure of the viscosity changes during the first few minutes of contact between "enzyme solution" (culture supernatant) and protein will therefore give an accurate estimate of the true proteinase concentration.

The gelatin and casein solutions were prepared as recommended by Northrop (1932) for standard methods, of peptic digestion. The actual viscosimetric determination of proteinase also followed Northrop's procedure (1932) with minor alterations.

The equation which was used to evaluate culture fluids for proteinase in this method was that suggested by Northrop (1932):

$$N = \frac{ts}{t\text{H}_2\text{O}} - 1$$

where N = the specific viscosity of the protein solution, ts = time of outflow of protein solution, $t\text{H}_2\text{O}$ = time of outflow of water from same viscosimeter.

The values of N were then plotted against the time elapsed after the enzymes and substrate solutions were mixed. The percentage change in viscosity for a given period was then interpolated from the curves. The zero point was determined by extrapolating on the curve obtained with a portion of enzyme solution previously boiled.

The per cent changes obtained by interpolation for a group of dilutions were averaged and the mean value determined for a digestion period of thirty minutes.   It will be seen from the data below that the per cent change caused by different dilutions of culture fluid varied directly with the reciprocal of the dilution used.   The error determined with a time period of thirty minutes was within 5 per cent.   When the period was taken at twenty minutes the error was consistently larger and therefore thirty minutes was chosen as the uniform point of calculation.   (See fig. 1.)

It is obvious from the data that the method allows definite appraisal of the proteinase content of a supernatant fluid.

TABLE 1

Per cent change caused in viscosity of 2.5 per cent gelatin solution, pH 7.0 by various amounts of proteinase of C. botulinum
Temperature of reaction 35.5° C.

DIGES-TION TIME	PER CENT CHANGE IN VISCOSITY				VALUE OF K				MEAN VALUE OF K	DILUTION RECIPROCAL CALCULATED MEAN K PER CENT CHANGE			
	Dilution reciprocal												
	2	3	4	5	2	3	4	5		2	3	4	5
minutes													
20	55.3	37.5	25.8	21.4	110.6	112.5	113.2	107	110.8	2.00	2.95	4.29	5.17
30	67.6	44.6	33	26.7	235.2	133.8	132	133.5	133.6	1.97	2.99	4.04	5.00

However there are certain points limiting the interpretation of any viscosity change of a protein by such an "enzyme solution." The most important is that by this method we have probably measured only one type of proteinase, "gelatinase," and have not measured the total proteinase activity.   Other proteinases such as that responsible for milk clotting should also be investigated, not only to determine their presence but also to check the validity of the viscosimetric method.   The investigation of the milk-clotting power of culture fluids of *C. parabotulinum* on purified enzyme preparations will form the subject of a subsequent paper.

It was next imperative that certain properties of the proteinase

be defined in order to determine the set of conditions under which it could be routinely evaluated in the future and, if possible, its relation to other proteinases.

Experiment 1.   Relation between proteinase activity and pH

Isoelectric gelatin was adjusted to the desired pH with HCl or NaOH.   The supernatant fluids from forty-eight-hour-old cultures were collected and brought to the proper pH value
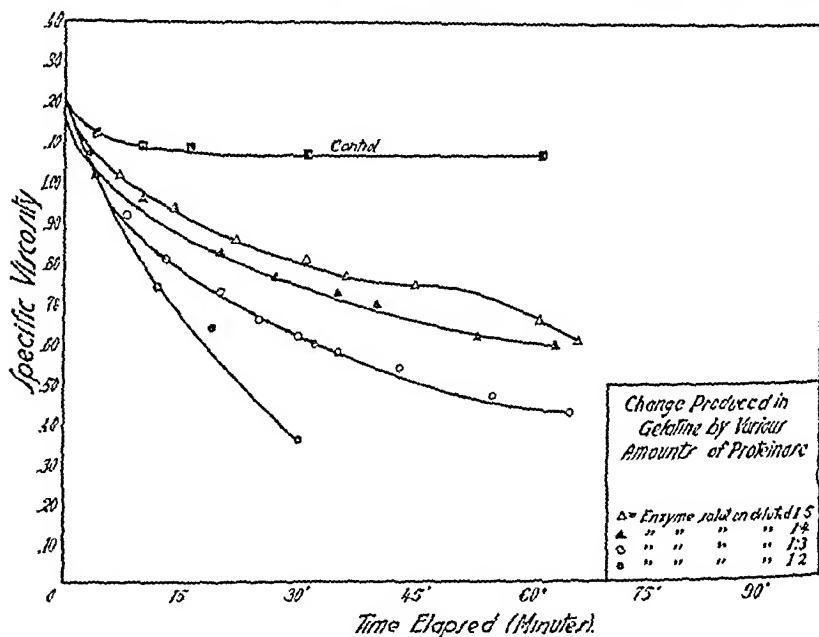

Fig. 1

before addition to the substrate.   All pH values were controlled electrometrically.

The greatest change in the viscosity of a gelatin solution was obtained in thirty minutes at pH 7.0.   In this respect the proteinase of *C. botulinum* differs from the animal proteinases, since the optimum pH for pepsin is about 2.5, for the cathepsins 3.7, for pancreatic proteinase 9, and for papain 7.4.   On the other hand, the value obtained here agrees well with those for *Clostridium histolyticum*, *Clostridium sporogenes*, and *Clostridium*

welchii as reported by Weil and Kocholaty (1937), and other bacterial proteinases.

We did not at this time attempt to answer the question con-

TABLE 2

Summary of the pH-activity data

pH	PER CENT CHANGE IN 30 MINUTES
8.0	59
7.0	71.5
6.0	62.6
4.95	11.4
4.0	0
3.0	0

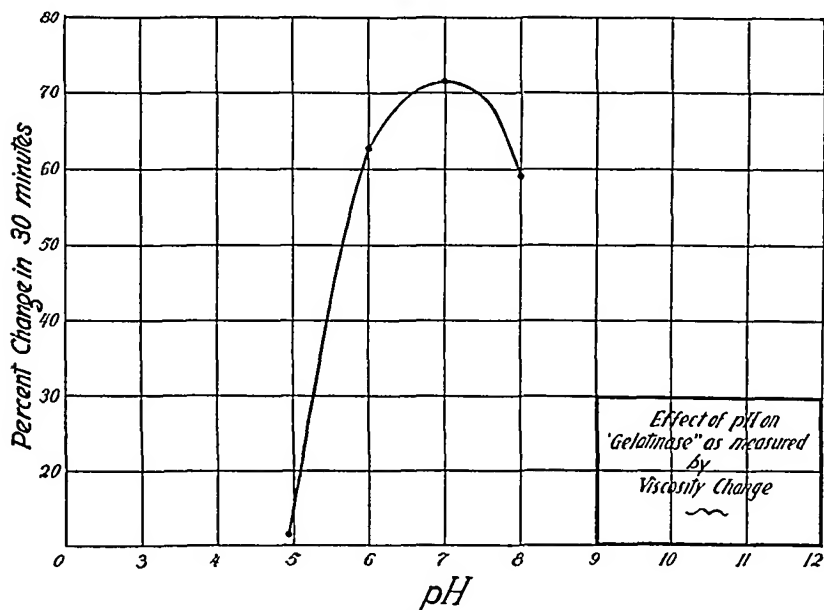Effect of pH on 'Gelatinase" as measured by Viscosity Change

Fig. 2

cerning the effect of pH on the enzyme and on the actual viscosity change. It is quite possible that our results in this section do not represent an effect of pH on the activity of the enzyme but rather the effect of pH on the rupture of the linkages in the

protein molecule. In other words, the change in viscosity per bond hydrolyzed may be greatest at a given pH. We may have been measuring this latter effect.

Having determined the "pH of maximal activity," it remained to characterize the proteinase by activations and inactivations.

a. The effect of cysteine. Cysteine hydrochloride solution, neutralized before testing, was added to aliquots of proteinase preparations so that there were final concentrations of 0.082 M and 0.0082 M cysteine per cubic centimeter enzyme solution. The mixtures were placed in a vacuum desiccator and incubated *in vacuo* for thirty minutes. The proteinase solution was then removed and its activity against gelatin determined viscosimetrically. All components of the digestion mixture were ad-

TABLE 3

Effect of cysteine

PROTEINASE PREPARATION	PER CENT CHANGE IN VISCOSITY OF GELATIN SOLUTION
(a) Untreated preparation..................................	64.2
(b) Heat-inactivated.......................................	0.0
(c) Treated with 0.0082 Mols cysteine......................	64.2
(d) Treated with 0.082 Mol cysteine.......................	52.6

Inactivation by cysteine = 11.6 per cent.

justed to pH 7.0 electrometrically. Controls were composed of (*a*) suitably diluted active proteinase preparations and (*b*) boiled enzyme plus 0.082 M cysteine. The results are presented in table 3 and figure 3. Unlike the proteinase of *C. histolyticum* (Weil and Kocholaty, 1937) cysteine inhibited that of *C. botulinum*. This effect, plus the action of cyanide to be discussed subsequently, indicates that the proteinase of *C. botulinum* is not of the papainase type.

b. The effect of sodium cyanide. Solutions of sodium cyanide in M/15 phosphate buffer, pH 7.0, were prepared so that there were 0.2 mol and 0.02 mol cyanide salt per cubic centimeter. Equal volumes of proteinase preparation and cyanide solution were incubated at 37°C. for thirty minutes, after which aliquots

were removed to gelatin solution and tested viscosimetrically. Control tubes included (a) heat-inactivated enzyme solution containing 0.2 mol of sodium cyanide per cubic centimeter, (b)
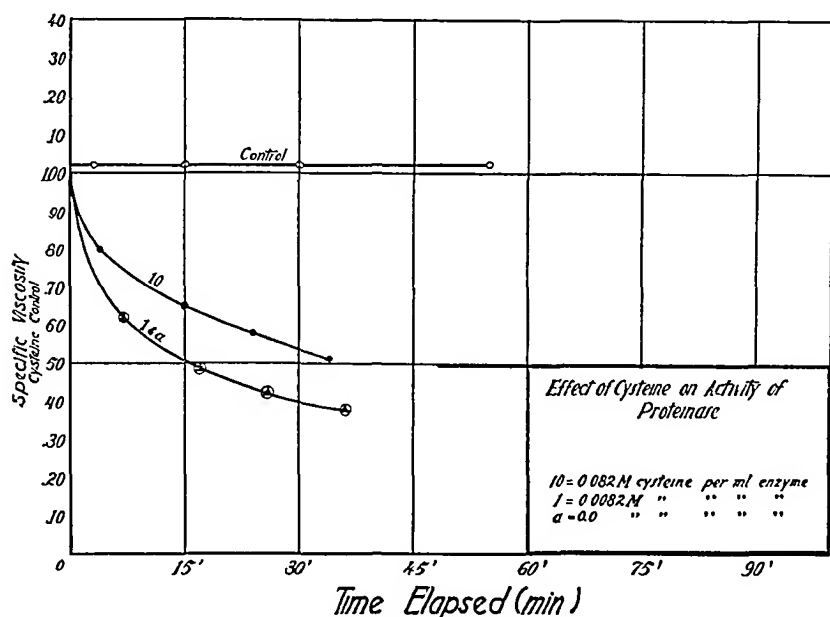

FIG. 3

TABLE 4

Effect of sodium cyanide on proteinase

PROTEINASE PREPARATION	PER CENT VISCOSITY CHANGE
(a) Untreated	45.3
(b) Heat Inactivated plus 0.1 Mol cyanide	0 0
(c) Heat Inactivated	0.0
(d) Treated with 0.1 Mol cyanide per cc. proteinase	0 0
(e) Treated with 0.01 Mol cyanide per cc. proteinase	45.3

Inactivation by 0 1 Mol sodium cyanide = 100 per cent.

heat-inactivated enzyme suitably diluted with distilled water, and (c) untreated enzyme suitably diluted. All pH values were checked before the mixtures were placed in the viscosimeter.

A total inhibition of the proteinase preparation by 0.1 mol sodium cyanide was observed, at pH 7.0.

Altering the pH to 5 and 9 during the cyanide inactivation did not change the effect, as Lawrie (1937) had observed to be the case with the proteinase of *Glaucoma piriformis*. The results obtained in this work are also contradictory to those of Maschmann (1937) who based his proteinase determinations on amino
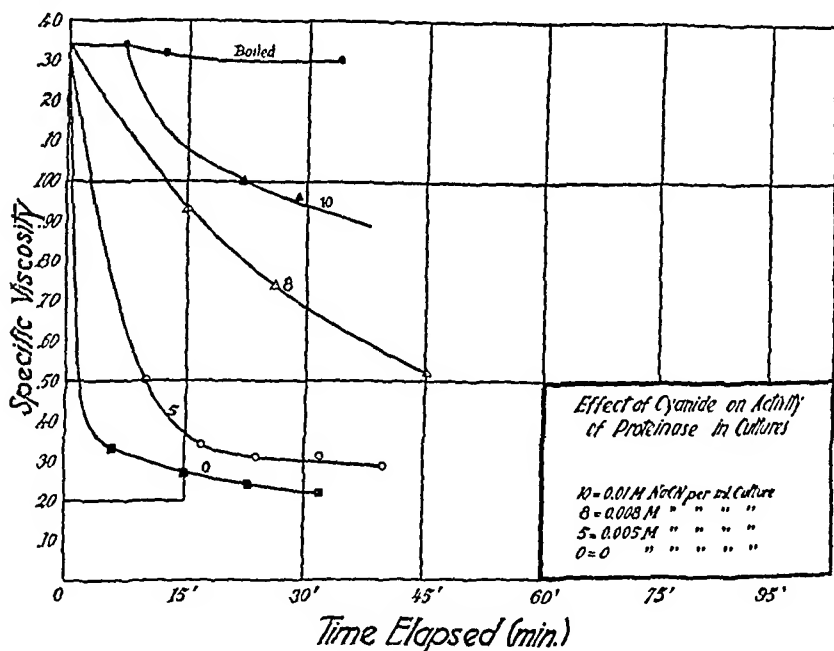

FIG. 4

group titrations. He found that M/100 hydrogen cyanide and M/250 cysteine activated the proteinase of *C. botulinum*.

Copper sulfate in the amount of 6×10^{-5} mol per cubic centimeter of proteinase also showed a complete inactivation viscosimetrically, both in air and *in vacuo*, in this series of experiments. The *in vacuo* tests were carried out to check the possible action of copper in catalyzing the uptake of oxygen or the oxidation of the proteinase.

It must be observed that the fundamental difference in the

methods which were used in these experiments and by Masch-
mann to determine "proteinase activity" may well be the cause
for the divergence of results. The viscosimetric method is one
predominantly involving a physical change in the micelle struc-
ture of the protein substrate. It certainly has been proven
that amino nitrogen or carboxyl group titrations measure the
entire proteolytic activity and not the action of the proteinase
alone. Hence the seemingly contradictory results on inactivation
phenomena are not strictly comparable.

c. The effect of hydrogen peroxide. To determine whether the
active group on the proteinase of *C. botulinum* was of sulfhydryl
nature, the action of hydrogen peroxide was tested. It was
conceivable that the peroxide might oxidize such a group which

TABLE 5

Effect of hydrogen peroxide

PROTEINASE PREPARATION	PER CENT VISCOSITY CHANGE
Untreated, dialyzed..	45
Heat Inactivated, dialyzed.................................	0.0
Treated with 0.1 cc. peroxide.............................	34.3
Treated with 1.0 cc. peroxide.............................	31.2

Inactivation by peroxide = 13.8 per cent.

could be reactivated by the addition of hydrogen sulfide to reduce
the action.

Five-cubic-centimeter aliquots of a 30-hour-old supernatant
fluid at pH 7.0 received 0.1 and 1.0 cubic centimeter amounts
of a 30 per cent hydrogen peroxide solution. The mixtures were
incubated one hour at 37°C. and then dialyzed for one hour
against distilled water in cellophane tubing to remove the
peroxide. One-cubic-centimeter amounts were tested on gelatin
solution. Control tubes checked the effect of (a) dialysis on the
enzyme and (b) boiled dialyzed proteinase.

Attempts to reactivate the peroxide-treated proteinase solu-
tion by hydrogen sulfide and thioglycollic acid were made but
were not successful. It appeared that the peroxide exerted an
irreversible inactivating effect.

The ability to decrease the viscosity of gelatin solutions was paralleled by similar alterations in casein solution. However, in this respect quantitative differences were noted, namely in the amount of proteinase solution required to cause such a change. It was found that the concentration of the enzyme, considered as per cent of the total volume of the digestion mixture, had to be raised to 40 per cent for casein digestion, whereas with gelatin it amounted to 16 per cent.
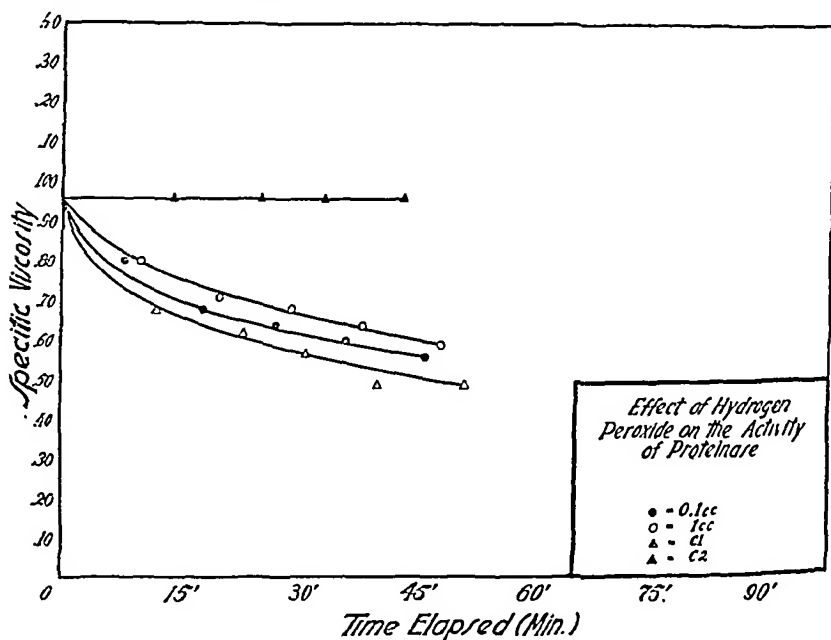
FIG. 5

The curves obtained with casein also lack the large initial change in per cent viscosity (0–15 minutes) so characteristic of gelatin digestion. A typical determination with casein is presented.

Three cubic centimeters of a 5 per cent casein solution at pH 7.0 plus 2 cc. of a proteinase preparation were tested in the usual manner. The control was composed of a boiled proteinase preparation.

It will be seen from the curves that the proteinase was able

to accomplish a 46.2 per cent change in the viscosity of the casein within thirty minutes.

Experiments on pH-activity relations, cysteine, cyanide and peroxide effects were carried out leading to the same results obtained with gelatin.

Edestin was also acted upon by the proteinase of *C. botulinum*. However, the results with this protein were not as clear cut as with gelatin or casein owing to the relative insolubility of the protein at the pH of optimum activity. Minor variations in the pH of the digestion mixture frequently caused minute flakes of edestin to form in the viscosimeter. As a result, most of the experiments on edestin had to be carried out around pH 8.0. The data at this point were however comparable to those for gelatin at the same pH.

To determine the extent of digestion of the protein of which the proteinase was capable, it was decided to use Willstätter and Waldschmidt-Leitz's alcoholic titration of the amino group (1921). Although such a method does not estimate proteinase alone, but the total activity of all the proteolytic enzymes in the fluid, it was felt at this time that preliminary experiments on whole proteins would pave the way in standardizing this procedure for the determinations of the lower peptidases.

There are approximately three typical methods for estimating amino nitrogen. These are (*a*) the nitrous acid method of Van Slyke (*b*) the formaldehyde method, and (*c*) the alcoholimetric titration. None is entirely satisfactory. The Van Slyke nitrous acid method is tedious, fortuitous errors arise from frothing and reducing agents in solution, and it gives faulty values for several amino acids and peptides. [Richardson, 1934.]

The formaldehyde and alcohol titrations are rapid and were specifically developed for enzyme and protein chemistry. Furthermore, with a glass electrode available the end points are not difficult to attain, although they are on the alkaline side in a range where the electrode works with less precision. With control solutions treated identically, the values are only used in relation to each other and approximately the same electrode

error obtains in both the test and control series. The error of the electrode in this region (10.5) is equalled by the error in the colorimetric method.

TABLE 6

Alkalimetric determination of pH-activity curve

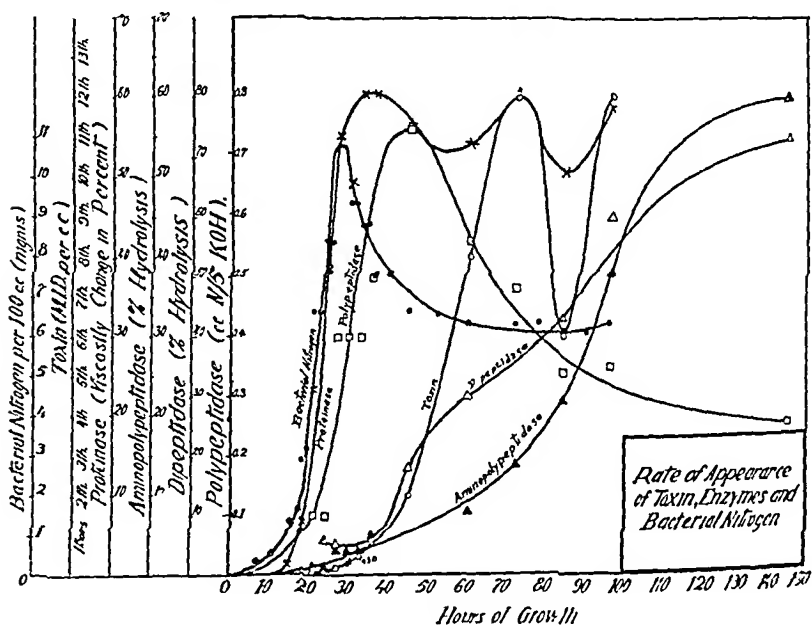| pH OF PROTEINASE PREPARATION	N/5 KOH	PER CENT CHANGE (BASED ON CHANGE AT pH 7 [5])
	cc.	
5.0	0.2	36.3
6.0	0.32	58.1
7.15	0.55	(100.) (arbitrary)
8.0	0.23	41.8
9.15	0.19	34.5

FIG. 6

The presence of phosphate buffer salts in the medium which interfered with the formol titration, plus the possible stepwise procedure in the alcoholic method, made it more profitable to use the latter method.

For completion the following experiment, relating activity to pH range, is presented.

Fifteen cubic centimeters of gelatin solution adjusted electrometrically with hydrochloric acid or sodium hydroxide to the desired pH were mixed with 3 cc. of supernatant fluid, also adjusted to proper pH value, and incubated 18 hours at 37°C. Controls consisted of boiled proteinase preparations or, in other cases, aliquots of the test mixture titrated immediately on mixing. The pH was adjusted to 7.0, and absolute alcohol added until the final alcohol concentration was 90 per cent. The mixtures were then titrated with N/5 KOH in 90 per cent alcohol to pH 10.5 by means of the glass electrode.

Comparison between the shape of the curve obtained by alkalimetric determination and that obtained viscosimetrically reveals an identity of optimum points but also shows in the former case a more gradual loss of activity on the alkaline side.

THE POLYPEPTIDASES

a. "Polypeptidase" activity was determined by using a 1 per cent Witte's peptone solution, at pH 7.4. Eight cubic centimeters of the peptone solution were mixed with 4 cc. of the supernatant fluid, and a 5 cc. aliquot withdrawn immediately for the control titration. The remainder of the digestion mixture was incubated at 37°C. and a 5 cc. aliquot titrated.

b. Aminopolypeptidase activity was determined with d,-l-leucylglycyl-glycine. A M/10 solution was prepared and adjusted to pH 7.8; 4 cc., containing 0.0004 mol, were mixed with 2 cc. supernatant fluid. A 3 cc. aliquot was removed immediately for the control titration and the remainder incubated at 37°C. for 18 hours.

c. Carboxypolypeptidase activity was determined with chloracetyl-l-tyrosine as substrate. A M/25 solution was used, of which 10 cc., containing 0.0004 mol, at pH 7.8, was mixed with 4 cc. supernatant fluid. An aliquot of 7 cc. was removed immediately for the control titration and the rest was incubated 18 hours at 37°C.

d. Dipeptidase was determined with d,l-leucyl-glycine and

glycyl-glycine as substrates. M/10 solutions were prepared, of which 4 cc., containing 0.0004 mol, at pH 8.0, were mixed with 2 cc. supernatant fluid. A 3 cc. aliquot was removed immediately for the control titration and the remainder titrated after 18 hours at 37°C.

e. Tri-glycine splitting ability was determined with diglycyl-glycine, using M/10 solution, at pH 7.8. The digestion mixture contained 4 cc. (0.0004 mol) substrate plus 2 cc. supernatant fluid. The usual control was titrated and the test incubated 18 hours at 37°C.

It was to be expected from the growth of *C. botulinum* in commercial peptones that a "polypeptidase" would be present in the

TABLE 7

Peptidase activity of a 48-hour-culture supernatant fluid

ENZYME DETERMINED	TITRATION VALUE (CC. N/5 KOH)	PER CENT OF THEORETICAL HYDROLYSIS
1. "Polypeptidase" (48 hrs.)...............	0	0
(24 hour culture)....................	0.8	
(72 hour culture)....................	0.25	
2. Aminopolypeptidase....................	0.03	3.0
3. Carboxypolypeptidase.................	0.0	0
4. Dipeptidase:		
(a) Leucyl glycine....................	0.06	6
(b) Glycyl glycine....................	0.0	0
5. Tripeptidase (Diglycyl glycine)........	0.0	0

extracellular system. The significance of this hydrolysis is of course not clear, owing to the uncertainty of the chemical nature of the substrate, and to the fact that proteinases also attack peptones. The ability of the supernatant to decompose "Proteose Peptone" would indicate the ability of the organism to produce peptidases acting on polypeptides of more than three amino acids in the chain.

The lack of ability to decompose chloracetyl-l-tyrosine, indicating the absence of a carboxypolypeptidase, was confirmed by testing supernatant fluids every 24 hours over a period of 240 hours at pH values from 4 to 10. At no time was there any indication of such an enzyme in the extracellular system.

The presence of an aminopolypeptidase was confirmed repeatedly. It was also determined that after 5 to 6 days the hydrolysis would exceed 50 per cent, indicating that both optically active forms were hydrolysed. This enzyme in supernatant fluids of *C. botulinum* was also found by Maschmann and he observed that it was specifically inactivated by cysteine and cyanide.
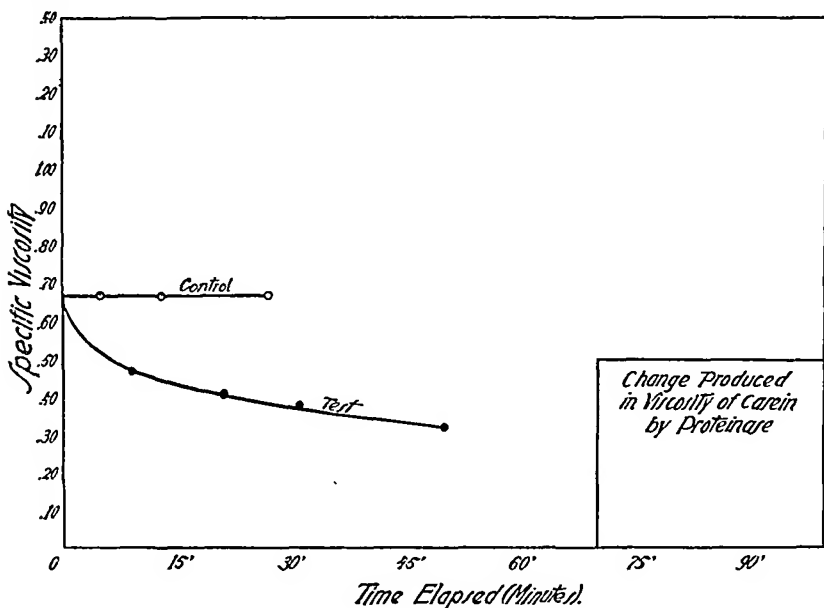
FIG. 7

The presence of a dipeptidase was confirmed, and in the case of *Clostridium parabotulinum*, it was also found that peptides containing only molecules of glycine were not hydrolysed. Johnson and Peterson (1935 a, b) noted this same specificity in the case of the dipeptidase of *Aspergillus parasiticus*. It was observed that, in the case of *C. botulinum*, apparently both optically active forms were hydrolysed inasmuch as the per cent hydrolysis was often equivalent to more than 50 per cent.

The inability to hydrolyse diglycyl glycine further emphasized the specificity of the polypeptidases.

The optimum points for activity of the aminopolypeptidase and dipeptidase in the extracellular system were determined to be pH 7.8 and 8.0 respectively.

It was decided to postpone indefinitely further experiments on activation and inactivation procedures on these enzymes until a time-correlation between them and toxin production could be tentatively established.

The extracellular proteolytic system of *C. parabotulinum*, A, apparently consists at least of a "proteinase," which changes the viscosity of gelatin, casein and edestin, and aminopolypeptidase, a dipeptidase, and a "Polypeptidase." In this system there are no carboxypolypeptidases nor are there enzymes hydrolyzing chains of glycine molecules.

In the case of *C. botulinum* certain experiments have been reported of potential significance. Stark, Sherman and Stark (1928a, b) allowed sterile filtrates of cultures to act on sterile skimmed milk, purified casein, and yeast cells. It was found that when one part of toxic filtrate was mixed and incubated with 49 parts of milk a seven-fold increase in the M.L.D. per cubic centimeter content of the toxic mixture resulted. These results were confirmed and extended by Meyer (1928).

The apparent significance of the extracellular enzymes, plus the fact that no direct approach to this problem has been reported, where the enzymes have been materially inhibited and the toxin formation determined, gave impetus to the collection and presentation of relevant data. An examination of the proteolytic enzymes present in the surrounding medium during all phases of growth has been attempted.

Experimental

The first step taken was to determine the time of appearance of toxin and the extracellular proteolytic enzymes.

Experiment 1. Tubes containing 10 cc. of glucose-beef heart medium prepared as described in the introduction were inoculated with a suspension of dried spores. The inoculum was small enough so that no change in turbidity of the medium resulted. At stated periods tubes were removed from the series, centrifuged

to remove the organisms, and the latter removed and washed in saline. Before the final washing and centrifugalization, the organisms were transferred to conical micro-Kjeldahl centrifuge flasks. The sediment was then resuspended in 1 cc. distilled water.

The Kjeldahl method for determining nitrogen, modified by Pregl (1930), was used to determine the bacterial nitrogen. One cubic centimeter of concentrated sulfuric acid was added to the suspension, which also received a knife-point of a mixture of copper and potassium sulfate (1:2). It was also found advisable to accept Mueller's recommendation (1935) that 4 drops of 30 per cent hydrogen peroxide be added three times during the digestion. The distillations were carried out in an all-glass apparatus designed and made by Mr. Cummings of the University Chemistry Department.

On the supernatant fluid from the centrifuged cultures determinations of proteinase, aminopolypeptidase, dipeptidase, and "polypeptidase" were made.

Another aliquot of the same supernatant fluid was diluted in a series of 10-fold dilutions and the toxin concentration determined in terms of M.L.D. per cubic centimeter by mouse inoculation. Duplicate mice were used for each dilution. Five-tenths cubic centimeter of each dilution was inoculated intraperitoneally and the amount required to kill a 20 gram mouse in 48 hours determined as closely as possible.

The composite data for a typical run are presented in table 8.

It will be observed that maximum bacterial nitrogen is attained around the 27th hour, after which a slow decline occurs, accompanied by a visible decrease in turbidity of the culture fluid. The nitrogen values level off and remain fairly constant thereafter.

Toxin begins to appear as early as the 21st hour of growth, reaching a peak long after the bacterial nitrogen maximum, and, it will be noticed, during the phase of visible autolysis.

The proteinase parallels the bacterial nitrogen curve, and appears about 6 hours before toxin is detectable. On the other hand the lower peptidases do not appear in appreciable amount until long after the toxin has appeared. One would be led to

believe that, if any of the enzymes might bear a causal relationship the proteinase would bear further investigation.

Experiment 2. Sodium cyanide solutions were prepared in M/15 phosphate buffer at pH 7.0. Ten-cubic-centimeter tubes of glucose-beef heart medium were adjusted to contain varying

TABLE 8

GROWTH	BACTERIAL NITROGEN PER 100 CC.	TOXIN (M.L.D. PER CC.)	PROTEINASE (VISCOSITY CHANGE)	AMINOPOLY-PEPTIDASE	DIPEPTIDASE	POLYPEPTI-DASE (CC. N/5 KOH)
hours	*mgm.*		*per cent*	*(per cent hy-drolysis)*	*(per cent hy-drolysis)*	
7	0.33	0	0	0	0	0
	0.50					
15	1.35	0	1.3	0	0	0
	1.68					
18	2.94	0	6.9	0	0	0
	3.20					
21	6.65	20	23.4	1	0	0.1
	6.65					
24	8.40	80	38.6	1	6	0.1
	8.39					
27	10.8	160	55.1	3	5	0.4
30	9.38	400	49.4	3	3	0.4
	9.38					
33	8.82	480	60.5	3	3	0.4
	8.86					
36	7.56	800	60.5	5	6	0.5
	7.59					
45	6.65	2,000	57		18	0.75
	6.58					
60	6.33	8,000	54.1	8	30	0.56
72	6.30	12,000	60	14		0.48
	6.37					
84	6.16	6,000	50.5	22	43	0.32
	6.11					
96	6.37	12,000	58.8	38	60	0.35
144				60	73	0.25

amounts of the cyanide salt and were then inoculated with a heated spore suspension. Sets of ten tubes per concentration of cyanide were used.

In 30 hours, tubes containing 0.01 mol, and less, cyanide per cubic centimeter medium showed growth equal to that in the

control tubes containing no cyanide. Tubes containing more than 0.01 mol sodium cyanide per cubic centimeter medium eventually showed growth but the appearance of this growth was sometimes delayed for 8–10 days. 0.02 mol was the upper limit of cyanide concentration (per cubic centimeter medium), above which no growth ever occurred.

Only tubes showing growth as soon as the control series (with no cyanide) were tested inasmuch as it was desired to simulate natural conditions as much as possible.

The proteinase tests were carried out in the usual manner with gelatin solution, at pH 7.0.

TABLE 9

Effect of sodium cyanide on production of proteinase

PROTEINASE PREPARATION	PER CENT CHANGE IN VISCOSITY
1. Untreated culture...	83.5
2. Secreted in presence of 0.005 Mol per cc.................	77.5
3. Secreted in presence of .008 Mol per cc..................	52.3
4. Secreted in presence of 0.01 Mol per cc.................	28.3
5. Heat-inactivated...	0.0

"Inactivation" by sodium cyanide in medium:
0.005 Mol.................................... 7.2 per cent
0.008 Mol....................................38.3 per cent
0.01 Mol....................................66.2 per cent

The toxin determinations were made for M.L.D. per cubic centimeter of medium. Inasmuch as the tubes with 0.005, 0.008 and 0.01 mol sodium cyanide allowed growth at a rate equal to that of the control tubes, they were chosen for assay. The proteinase tests are presented in table 9.

That the proteinase was not simply altered in its rate of appearance rather than in the total amount produced was tested by examining over a period of 96 hours the proteinase content of the medium. The maximal point was reached in every case around the 30th hour with no further increase.

If the extracellular proteinase is directly concerned with toxin production, then tubes containing 0.008 and 0.01 mol. sodium

cyanide per cubic centimeter of medium should show a decreased amount of toxin, around the 96th hour when it is usually maximum in this medium. Such did not prove to be the case, however, as the data in table 10 indicate.

Tubes in which the proteinase showed 60 per cent inhibition or reduction in concentration were inoculated in 10-fold dilutions into mice in the usual manner. Control mice received antitoxin to check the possible effect of the cyanide and heat-inactivated toxin to further check the natures of the killing agent. A third series of mice received the supernatant fluid from cultures grown in the absence of cyanide.

The complete similarly in picture between the cultures grown in the presence and absence of sodium cyanide was confirmed

TABLE 10

TOXIN DI-LUTION (0.5 cc.)	CYANIDE-TREATED CULTURES	UNTREATED CULTURES	"HEAT-KILLED" TOXIN	ANTITOXIN-PRO-TECTED
10^{-2}	Dead—18 hours	Dead—18 hours	No symptoms	No symptoms
10^{-3}	Dead—26 hours	Dead—28 hours	No symptoms	No symptoms
10^{-4}	Dead—45 hours	Dead—43 hours	No symptoms	No symptoms
10^{-5}	Dead—55 hours	Dead—50 hours	No symptoms	No symptoms
10^{-6}	Symptoms severe	Symptoms mild	No symptoms	No symptoms
10^{-7}	No symptoms	No symptoms	No symptoms	No symptoms

on several repetitions. It was therefore concluded that a 60 per cent reduction in the proteinase activity of the extracellular system does not cause a reduction in the toxin concentration detectable in this manner. It remains to test this assumption again on very much larger numbers of mice for statistical evaluation.

Experiment 3. The fact that the efficiency of the bacterial enzyme systems may exert a masking influence, and that the 40 per cent remaining activity of the enzyme may still account for toxin production was tested by examining the distribution of the proteinase in types of *C. botulinum.*

Four "B" types, two "A" types, a "C" and a "D" types were examined. The data are presented in table 11.

For comparison, strains 526 C, 237 B, D, and 63 B were selected

for toxin determinations.   Titrations of the supernatant fluid from 96-hour-old cultures in mice again failed to show any correlation with the proteinase activity as the data in table 12 indicate.

The cultures from Type D and strain 237 B show on one hand maximal deviation in enzyme activity and on the other a quantitative similarity in toxin-production ability.   Strain 63 B

TABLE 11

Proteinase distribution

STRAIN	PER CENT CHANGE IN VISCOSITY IN 30 MINUTES BY 1 CC. CULTURE FLUID	
	24 hour culture	48 hour culture
237 (B)	83.6	81.0
34 (B)	74.5	39.3
London (A)	63.9	73.4
63 (B)	59.0	58.3
69 (A)	54.0	
6 (B)	30.3	43.9
526 (C)	9.8	0.0
(D)	0	0.0

TABLE 12

Distribution data of proteinase and toxin

STRAIN	PROTEINASE ACTIVITY	M.L.D. PER CC.
237	83.6	200,000
526	9.8	20,000
D	0	200,000
63	59	20,000

showing a moderately strong proteinase belongs in the same class toxigenically speaking with 526 C, which is practically inert as far as protein-splitting ability is concerned.

It would appear therefore very probable that the residual activity of the extracellular proteinase in cyanide-treated cultures was not a factor in toxin production but more probably concerned the maintenance of the bacterial *"status quo."*

The question concerning the designation of a toxin as an exo-

toxin or an endotoxin and the implications arising from such terms have recently been brought to attention once more.

The neurotoxins of *Corynebacterium diphtheriae, Clostridium tetani* and *Clostridium botulinum* have been generally accepted as classical examples of exotoxins. It is certainly true, in addition, that these toxins possess characteristics which at once distinguish them from the endotoxins of the cholera vibrio and the meningococcus. However, the true state of affairs in designating these toxins as endotoxins or exotoxins has been aptly summarized by Topley (1933) in the statement that "although this broad distinction can be made by comparing typical endotoxins with typical exotoxins, our difficulties become great if we attempt to assign each bacterial product to its correct group. . . ."

The data on the time of appearance of diphtheria, tetanus and botulinus toxins show that these toxins do not appear until the cells have reached the maximum growth phase, and, in fact, as our results show, botulinus toxin appears maximally during the phase when most of the cells are autolyzing.

The results of Eaton (1936a, b, 1937) have shown almost conclusively that the diphtheria toxin is a protein, which is heat-coagulable and which can be denatured by acids. Pappenheimer (1937a, b, c) has studied the purification of diphtheria toxin in a far simpler medium and also believes that the diphtheria toxin is a protein. Both investigators have isolated this toxic protein from media in which no protein tests were given before inoculation. The period of incubation before the toxin was harvested was around five to six days, enough time to elapse for considerable lysis of cells if reference is made to a paper by Moloney and Hanna (1921) and to the more recent and accurate studies of Mueller (1935).

The evidence indicates that diphtheria toxin is formed inside of the cells and is set free on the death and destruction of the cells.

Our results with botulinus toxin also point to its intracellular formation on the basis of the rise in M.L.D. when visible autolysis occurs. Furthermore, the difficulty of obtaining atoxic sus-

pensions of diphtheria and botulinus bacilli by washing is well-known. With diphtheria it is possible to produce anti-bacterial sera which are not antitoxic, as Eaton (1936b) succeeded in demonstrating. However, in the case of botulinus, Thom, Edmondson and Giltner (1919), Burke, Elder and Pischel (1921) and Nelson (1927) have stated that it is impossible to prepare botulinus bacilli or spores suspensions free of toxin, even after fifteen to twenty washings. In fact, Nelson (1927) found that a mass of botulinus bacilli could be extremely toxic despite the atoxicity of the broth fluid in which they had grown.

It would appear therefore that, with *C. botulinum*, one is dealing with an endotoxin, or at least with a toxin the locus of production of which is inside the cell. This change in conception does not in fact complicate the toxin make-up of bacteria because Boivin, Mesrobeaunu, Topley *et al* have shown that the classical endotoxins are very closely related, if not identical with, the somatic antigen. The toxin production of bacteria can therefore be pictured as occurring entirely within the cell. The true non-antigenic endotoxins may be considered intimately bound or identical with the soma whereas the characteristic antitoxinogenic toxins, also produced intracellularly, may be considered as substances to which the cell membrane is impermeable during the life of the cell, and which are distinct from the somatic antigens of the cell.

SUMMARY

1. The extracellular proteolytic system in relation to toxin production was studied. It was found that a "proteinase," acting on gelatin and casein was secreted into the medium. This enzyme acted optimally at pH 7.0. It was inactivated by sodium cyanide, cysteine, hydrogen peroxide and copper salts.

2. The lower members in the proteolytic system included a polypeptidase acting on Witte's peptone, an aminopolypeptidase, hydrolyzing leucyl-glycyl glycine and a dipeptidase, hydrolyzing leucyl glycine. The optimal pH values for these enzymes was 7.8–8.0.

3. Experiments on rate of appearance revealed that the

proteinase appeared first in the culture fluid, paralleling the trend of bacterial nitrogen. It also appeared at least six hours before the toxin could be demonstrated.

4. The polypeptidases and dipeptidase appeared simultaneously with toxin but, whereas the toxin reached its maximum around the ninety-sixth hour, these enzymes slowly increased during the first six days.

5. When, during the normal growth of the organisms, the proteinase was inactivated by the presence of sodium cyanide in the medium, the production of toxin was unchanged and unaltered.

6. Various types of *Clostridium botulinum* showed no correlation between the extracellular proteolytic enzymes and toxin production. Types "C" and "D" produced large amounts of toxin, comparable with the proteolytic type "A" in the absence of any detectable quantities of proteinase. Strains of type "B" on the other hand produced amounts of proteinase comparable to that of type "A" but produced approximately one-tenth as much toxin.

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METABOLIC STUDIES OF A NON-HEMOLYTIC STREPTOCOCCUS[1]

J. W. KING, J. C. GAREY, AND M. A. FARRELL

Division of Bacteriology, The Pennsylvania State College, Pennsylvania

INTRODUCTION

The nutrition of the non-hemolytic streptococci, which occur often as human and animal pathogens, has been studied relatively little. A member of this group of streptococci was selected because of earlier studies using this organism, and also because it was felt that information obtained with this culture might aid in the solution of the nutritional requirements of the more fastidious hemolytic streptococci.

Earlier workers on the metabolism of streptococci have confined themselves almost wholly to hemolytic strains. Kobayshi and Nishikawa (1921) reported that a hemolytic streptococcus studied by them responded to the addition of .005–.5 per cent cystine and .02–.2 per cent histidine hydrochloride to sheep-serum media.

Gordon and McLeod (1921) studied the effect of amino acids on a number of organisms, including several hemolytic streptococci. They reported high concentrations of amino acids to be very readily inhibitory to the growth of these bacteria.

Mueller (1922a) showed that casein acid digests and beef infusions contained a growth factor for hemolytic streptococci. He also (1922b) showed that the factor could be removed by absorption of the digests or infusions on charcoal. Tryptophane, tyrosine, cystine, and histidine would not substitute for this factor.

Freedman and Funk (1922a-b), using a hemolytic streptococcus as a test organism, also observed this growth factor in a number of substances, including beef-'muscle' and beef-heart infusions, peptone and brewer's yeasts.

Whitehead (1924) also reported the presence of a growth factor for hemolytic streptococci in the Dakin butyl-alcohol-insoluble fraction of casein acid digests.   He studied various amino acids and reported (1926) that inorganic phosphates were probably more important than amino acids in the nutrition of streptococci.

Thompson (1929) observed the effect of vegetable and beef heart extracts on the growth of hemolytic streptococci which were not able to grow on nutrient broth.   The growth-promoting power of the extracts was destroyed by heat.

Krasnow and Rosenberg (1929) and Krasnow and Gies (1929) studied synthetic media, supplemented with such simple sources of nitrogen as amino acids and ammonium salts.   In the 671 and 441 media studied respectively, none gave growth past the second subculture, with the strains of dental streptococci used.

Farrell and Thomas (1932) using a gamma-type streptococcus called *Streptococcus rheumaticus* observed survival times on various synthetic media, using ammonium salts, amino acids, and amines as the nitrogen sources.   In general, they found that phenylalanine, leucine, cystine, glutamic acid and histidine were beneficial to the survival of the culture.

Koser and Saunders (1935) isolated a substance from beef extract which stimulated the growth of streptococci and other bacteria.   Very small amounts of this factor, when added to a synthetic base, fostered growth.

Smith (1938a-b) observed that the production of haemolysin by hemolytic streptococci was strongly affected by changing the concentration of peptone in the medium.   Excess peptone, especially the dialysable fraction, inhibited the production of haemolysin.

Hutner (1938) has studied the effect of various fractions of casein acid digests obtained by precipitation and by other methods on the growth of the hemolytic streptococci.   He showed that the sulfur-bearing amino acids were very important in streptococcal metabolism.

Koser, Finkle, Dorfman, Gordon and Saunders (1938) attempted to cultivate a hemolytic streptococcus in a synthetic medium of salts, amino acids, etc.   Various factors were added, such as tissue extracts, the sporogenes vitamin and the growth

factor for the propionic acid bacteria. No growth was observed with any of these substances.

It was the purpose of the present study to investigate the growth-promoting ability of casein acid hydrolysates and the fractions of such hydrolysates for a representative pathogenic, non-hemolytic streptococcus. In addition, upon learning which fraction or fractions stimulated growth the amino acids known to be present in such fractions were studied individually to determine which of these substances is most essential to the nitrogen metabolism of this streptococcus.

PROCEDURE

The casein acid hydrolysates used in this study were prepared and fractionated according to the method of Dakin (1918). Briefly, this consisted of digesting a technical grade of casein in a refluxing condenser with 30.0 per cent sulphuric acid. The sulphuric acid was removed quantitatively with barium hydroxide after diluting the digest to several volumes with distilled water. The neutralized digest was concentrated *in vacuo* to a volume of about 500 cc. per 100 grams casein used. The digest thus prepared was allowed to stand in the refrigerator over night. The more insoluble tyrosine and part of the leucines were precipitated and removed by filtering. The remaining digest was made up to 500 cc. volume per 100 grams casein. It was then fractionated in a Kutscher-Steudel extractor with normal butyl alcohol. The resulting three fractions, when purified according to Dakin consisted of the following groups of substances:

Fraction A: monamino, monocarboxylic amino acids, less tyrosine and a part of the leucine isomers. This fraction is extracted in butyl alcohol and precipitates out as a white powder.

Fraction B: proline and hydroxy proline which remains in solution in the butyl alcohol in the extraction flask.

Fraction C: the butyl-alcohol-insoluble fraction, made up of the diamino and dicarboxylic amino acids and the heterocyclic acids. Tryptophane is absent from this group as it is destroyed by acid digestion.

The fractions thus secured were sterilized by autoclaving at 15 pounds for 15 minutes.

The organism employed in this study, *Streptococcus rheumaticus*, Allen strain, was a representative strain of a non-hemolytic, pathogenic streptococcus which was secured from the Lister Institute. The organism was a very short-chained streptococcus; it grew well on the beef-infusion stock culture medium and poorly, or not at all, on 0.3 per cent beef-extract water. This strain had been carried in the stock culture collection on ground-meat medium. During the period of this study the culture was carried on beef-infusion agar, pH 7.4–7.6, and transferred every other day over a period of six months before being used in this investigation. This was found necessary because inconsistent results were obtained when care was not taken to assure a young, active, uniform culture. Older cultures tended to become adapted to the basal medium and in many cases made duplication of results difficult.

The basal medium, to which the hydrolysate fractions and amino acids were added, was 0.3 per cent beef extract (Difco) water. (It was presumed that the carbon and mineral sources were supplied by this extract.) In all cases where the volume was altered by adding amino acid solutions, the basal medium was so prepared as to provide the proper final volume and concentration of all constituents. The reaction of these media was pH 7.4–7.6 and all supplementing materials were checked for proper pH values. The media containing digest fractions were prepared as indicated in the several tables. When a medium is shown as containing 5.0 per cent of a fraction, it is to be interpreted that each 10 cc.[2] of culture medium contains 0.5 cc. of the fraction indicated. These media were sterilized by autoclaving at 15 pounds for 15 minutes. The amino acids used in this study were made up in solutions of varying concentrations (depending upon the solubilities of the acids), and sterilized by filtration. They were added in the required amounts, as later determined, to prepared and sterilized beef extract water.

All estimations of growth were made for final recording after culturing the organism on the medium under consideration three or four times. It was assumed that the third subculture should

[2] All media were dispensed so that each culture tube contained 10 cc. of medium.

give growth which was not affected by food materials carried over with the inoculum from the infusion-broth culture medium. Inoculations were made with a straight nichrome needle. In preliminary investigations growth was estimated by visual estimations of turbidity and by colorimetric and volumetric micro-Kjeldahl procedures. These methods were not satisfactory. The first, because it involved excessive dependence upon the human factor, could hardly be considered as more than semiquantitative. The second and third procedures are inaccurate because they both require that the cells be separated from the culture medium. This is very difficult when dealing with small amounts of culture medium and is especially difficult with streptococcal cells of this type, which do not form a pellicle or pack well. For these reasons growth was measured by using a photoelectric colorimeter to determine the turbidity of cell suspensions.[3]

The turbidity of each culture was determined in triplicate against the uninoculated controls. The resulting readings were treated as ordinary colorimetric values and the index of growth produced was secured by dividing the reading of the culture cup into the reading of the uninoculated culture medium control. By reducing the denominator of this fraction to one, the ratio or index thus secured was then used as a value to compare with a similar value secured with the beef-extract water culture. The resulting expression:

$$\frac{\text{Index of growth of any culture medium}}{\text{Index of growth of the beef-extract water}} = \text{growth index}$$

was used as a means of comparing growth-promoting ability.[4]

[3] Since the turbidity produced by the growth of the strain used was very even it was felt that if it were possible to measure turbidity by some mechanical means a relatively satisfactory method of estimating growth could be devised.

[4] In order to make clear just how this growth index was derived an example is given:

	Colorimetric readings of uninoculated control cup	of inoculated medium
Medium X....................................	21.0	10.0
Beef extract..................................	12.0	10.0

Growth index of medium X = 21/10 = 2.10.
Growth index of beef-extract medium—12/10 = 1.20.
Growth index as recorded in final column of the table then was: 2.10/1.20 = 1.75.

A comparison of these values with micro-Kjeldahl determinations of bacterial nitrogen showed the following correlation:

Growth index	Mgm. bacterial nitrogen per 100 cc. of culture
2.4	73.4
2.0	66.0
1.7	54.2
1.5	33.4

These indexes are believed to be a fairly accurate representation of the growth-promoting or inhibiting ability of the substances under investigation.

TABLE 1

The effect of different concentrations of unfractionated casein hydrolysate on the growth of Streptococcus rheumaticus

DIGEST PER 100 CC. MEDIA	CONC. BEEF EXTRACT	FIRST CULTURE	SECOND CULTURE	THIRD CULTURE	GROWTH INDEX
cc.	per cent				
None	0.3	+*	+	+	1.00
2.5	0.3	++	++	+	1.37
5.0	0.3	++	++	+	1.22
7.5	0.3	++	++	++	1.79
10.0	0.3	++	++	++	1.79
15.0	0.3	++	++	++	1.53
20.0	0.3	++	++	++	1.71
25.0	0.3	+++	+++	++	1.41

* — = no growth, + = trace of growth, ++ = poor growth, +++ = good growth.

THE GROWTH-PROMOTING ABILITY OF CASEIN HYDROLYSATES

In order to determine the influence of casein hydrolysates on the growth of *Streptococcus rheumaticus*, media were prepared containing concentrations of this hydrolysate up to 25 cc. per 100 cc. of basal medium. The inoculation and subculture procedure used in this and the remaining experiments in this paper was as follows: The media were inoculated from a 48-hour infusion agar culture of the test organism. After 48 hours incubation at 37°, subcultures were made into the respective media used. A third subculture was made at the end of another 48 hours incubation

from the second culture. All three cultures were examined for growth by visual estimation of turbidity after 48 hours incubation, and the third culture was also examined by means of the photoelectric colorimeter. The results are shown in table 1.

Table 1 shows that there is a marked stimulation in the growth of *Streptococcus rheumaticus* when reasonably small amounts of casein hydrolysates are added to the basal medium. While the best growth occurred in basal media containing 7.5 and 10.0 per cent of the hydrolysate, the values on either side of these two percentages are not great. Other hydrolysate preparations showed a higher growth index when 5.0 per cent was employed and for other studies in this paper it was decided arbitrarily to use the five cubic centimeter portion of the whole hydrolysate per hundred cubic centimeters of basal medium.

THE GROWTH-PROMOTING PROPERTIES OF THE THREE FRACTIONS OF THE CASEIN ACID HYDROLYSATE

In order to determine the influence of the individual fractions prepared by butyl-alcohol extraction on the growth of this streptococcus, 0.3 per cent beef-extract water media were prepared, supplemented with the individual fractions, alone and in combination with each other. The results are shown in table 2.

Table 2 shows the influence of the three fractions of the casein acid digest on the growth of *Streptococcus rheumaticus*. From the data presented it is evident that the greatest growth stimulation occurrs when fraction C is used either alone or in combination with fraction A. Fraction A alone stimulates growth of this streptococcus but slightly, while fraction B either alone or in combination with either or both of the other fractions inhibits growth. While the use of fractions A and C gives almost as much growth as the same per cent of the unfractionated hydrolysate, the use of all three fractions gives but a very slight growth stimulation. The inhibitory substances present in the B fraction may account for the difference.

Since the C fraction, containing diamino and dicarboxylic amino acids, was the portion of the hydrolysate showing the greatest growth stimulation, it was decided to determine the

influence of the concentration of this fraction on the growth of this streptococcus. Table 3 shows the results of inoculating media prepared with increasing amounts of fraction C.[5]

TABLE 2

*The effect of the various hydrolysate fractions on the growth of
Streptococcus rheumaticus*

CONC. FRAC-TION A	CONC. FRAC-TION B	CONC. FRAC-TION C	FIRST SUB-CULTURE	SECOND SUB-CULTURE	THIRD SUB-CULTURE	FOURTH SUB-CULTURE	GROWTH INDEX
cc./100 cc.	cc./100 cc.	cc./100 cc.					
None			−	+	+	+	1.00
5.0			±	+	+	+	1.23
	5.0		−	−	−	−	0.99
		5.0	++	++	+	++	1.59
5.0	5.0		−	−	−	−	1.00
5.0		5.0	++	++	++	+++	1.83
	5.0	5.0	−	−	−	−	1.01
5.0	5.0	5.0	−	−	−	−	1.05
5.0*			++	++	+++	+++	1.85

* cc. of the unfractionated hydrolysate.

TABLE 3

*The effect of different concentrations of fraction C on the growth of the
test organism*

CC. OF FRACTION C PER 100 cc. OF MEDIUM	BEEF EXTRACT	FIRST SUB-CULTURE	SECOND SUB-CULTURE	THIRD SUB-CULTURE	GROWTH INDEX
	per cent				
None	0.3	−	+	±	1.00
3.0	0.3	+	+	+	Lost
5.0	0.3	+	+	+	1.23
10.0	0.3	+	+	++	1.26
15.0	0.3	+	+	++	1.21
20.0	0.3	+	+	±	1.52
30.0	0.3	+	+	±	1 38

The data in table 3 show that under the conditions of this study 20 cc. of digest per 100 cc. of medium gives the greatest amount of growth. Since we were not interested in the maximum

[5] As a check to see whether concentration of Fraction A would affect its growth promoting powers, a series similar to this C series was prepared with the A fraction. The results showed little, if any, stimulation of growth with the A fraction.

cell growth but rather in a plentiful supply of the growth factors under consideration 5.0 per cent of the C fraction was used in subsequent studies.

GROWTH-INHIBITORY PROPERTIES OF THE B FRACTION

Previous experiments showed that the B fraction inhibits the, growth of the test organism. This property was present in most, but not all, of the digests prepared and studied. As it was never present in the hydrolysate before butyl-alcohol extraction, it must have arisen as the result of the fractionating process or the subsequent purification of the fraction from the butyl alcohol.

TABLE 4

The effect of various concentrations of fraction B on the growth of Streptococcus rheumaticus

CONC. OF B FRACTION	CONC. A AND C FRACTIONS	BEEF EXTRACT	FIRST SUB-CULTURE	SECOND SUB-CULTURE	THIRD SUB-CULTURE	FOURTH SUB-CULTURE	GROWTH INDEX
	cc./100 cc.	per cent					
None	5.0	0.3	++	++	++	++	1.00
0.25	5.0	0.3	+	++	+	+	0.89
0.50	5.0	0.3	+	++	+	+	0.89
1.00	5.0	0.3	−	−	±	±	0.92
2.00	5.0	0.3	−	−	+	−	0.46
3.00	5.0	0.3	−	−	+	−	0.50

In studying the influence of the B fraction on the growth of this streptococcus, various percentages of this fraction were added to a basal medium consisting of the usual 0.3 per cent beef extract water containing 5 per cent each of fractions A and C. The results are shown in table 4.

Table 4 shows how an increasing concentration of fraction B of a casein acid digest shows an increasing inhibition of the growth ·of this streptococcus. Commercial preparations of proline and hydroxy-proline, when employed separately, or together with the basal medium, showed that these amino acids were not inhibitory to the growth of this organism. Since these substances are not responsible for the inhibition noted with the B fraction, it is probable that some substances are set free or formed during the fractionating or purification process.

THE EFFECT OF AMINO ACIDS ON GROWTH OF

Streptococcus rheumaticus

A number of amino acids were added to the basal medium, separately, to determine their influence on the growth of this

TABLE 5

The influence of various amino acids on the growth of Streptococcus rheumaticus

AMINO ACID ADDED	GROWTH INDEX
Basal medium alone......................................	1.00
A. Aliphatic, monoamino, monocarboxylic acids:	
Glycine..	0.86
Alanine..	1.20
Alpha-amino isobutyric acid............................	0.90
Valine..	1.17
Norleucine..	0.91
Leucine...	0.90
Isoleucine..	0.88
Serine..	1.01
Alpha-amino hydroxy normal butyric acid.............	0.91
Cysteine..	1.07
Methionine..	1.09
B. Diamino, monocarboxylic acids:	
Arginine..	1.54
Lysine..	1.07
C. Monoamino dicarboxylic acids:	
Glutamic acid.......................................	1.20
Aspartic acid.......................................	0.90
Asparagine*...	1.11
D. Aromatic amino acids:	
Phenyl alanine......................................	1.07
Tyrosine..	1.00
E. Heterocyclic amino acids:	
Tryptophane...	0.83
Histidine...	1.24

* Aspartic acid amide.

organism. In view of the fact that fraction C of the hydrolysate induced the best growth of this streptococcus, the amino acids which are known to be in this fraction, arginine, lysine, histidine, aspartic acid, glutamic acid and tryptophane were studied. In addition, certain monoamino, monocarboxylic acids including the sulfur-bearing acids cysteine and methionine were

added.   Other substances believed by certain investigators to be important in the nutrition of animals were also included.   While different concentrations of amino acids were studied the data presented in the table below are only for media containing the equivalent of 2 mgm. of amino acids per 10 cc. of culture media.[6]

An examination of table 5 shows that many of the amino acids studied when added to the basal medium favored the growth of *Streptococcus rheumaticus*.   The greatest stimulation is apparent with the dibasic amino acid arginine which is present in the C fraction of the hydrolysate.   Other amino acids of this fraction such as histidine, lysine and glutamic acid also stimulated the growth of this organism whereas aspartic acid and tryptophane appeared to be inhibitory.

In the monoamino, monocarboxylic group of acids, alanine, valine and to a lesser extent methionine and cysteine, were the only substances which stimulated the growth of this strepto-coccus.   The remaining acids in this group including the leucine isomers, glycine and alpha-amino beta hydroxy N. butyric acid were apparently inhibitory.

Studies with combinations of amino acids showed little, if any, differences from the effect of adding amino acids separately.

DISCUSSION

In this study, dealing with the nitrogen metabolism of a non-hemolytic streptococcus, it is necessary to remember that the results are conditioned to a certain extent by the basal medium employed.   The scant data available indicate in meat extract the presence of certain nitrogenous constituents including cystine. More recently Hughes (1932) and Knight (1935) found that beef extract contains a "growth factor" said to belong to the order of "natural bases."   Knight (1937) showed that this growth factor could be completely replaced by synthetic preparations of nicotinic acid amide and vitamin B_{-1} (aneurin).   Therefore, the results which may be observed, when one amino acid is added to a basal medium of this nature, must be interpreted with caution.

[6] The concentration of 2 mgm. per 10 cc. of medium was selected because less than this amount failed to stimulate growth and more than this gave less clear-cut results.

This study showing that arginine and histidine, diamino acids, and glutamic acid, a dicarboxylic acid, and in addition alanine and valine of the monoamino group, stimulated the growth of *Streptococcus rheumaticus* shows a close similarity to the previously reported results of Maver (1930), Fildes and Richardson (1935), Fildes (1935), Mueller (1935) and Sahyun et al. (1936) on the amino acid requirements of other organisms.

The inhibitory effect of tryptophane on the growth of this streptococcus is interesting in view of the ability of other bacteria to synthesize and use this amino acid, Fildes, Gladstone and Knight (1933), Sahyun et al. (1936).

It is hoped that studies now in progress using the chemically known growth factors present in beef extract in place of the complex extract itself will give further information regarding the nutrition of these streptococci.

SUMMARY

1. Employing a photoelectric colorimeter as a means of estimating the turbidity produced by the growth of *Streptococcus rheumaticus*, it was found that the monoamino, monocarboxylic acid fraction of a casein acid hydrolysate does not support growth; and that the proline fraction inhibits growth; while the butyl-alcohol insoluble fraction and the unfractionated hydrolysate stimulates growth.

2. The inhibitory action of the proline fraction, even in low concentrations, was shown to affect the growth of this streptococcus. Such inhibition was not caused by the amino acids, proline or hydroxyproline.

3. A study of the amino acids known to be present in the butyl-alcohol insoluble fraction showed that arginine, histidine, and glutamic acid, in the order named, stimulated the growth of this organism. Lysine and aspartic acid had little appreciable effect and tryptophane an inhibitory effect on its growth.

4. A few monoamino, monocarboxylic amino acids were studied. Alanine, valine, and to a lesser extent cysteine and methionine, stimulated the growth of this organism.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MARYLAND BRANCH

GOUCHER COLLEGE, BALTIMORE, FEBRUARY 23, 1939

THE ANTIBACTERIAL EFFECTS OF THE ORGANIC MERCURIAL COMPOUNDS. With special reference to their use as germicides for the sterilization of surgical and dental instruments. *John H. Brewer*, Hynson, Westcott & Dunning, Inc., Baltimore.

An improved centrifuge technique was described which was developed to determine whether the mercurials under optimal conditions are capable of destroying spores of anaerobes. The outstanding features of this technique are as follows: a sealed tube is used in which the organisms are exposed to the chemical agent for twenty-four hours, during which time the mercurial spore mixture is agitated constantly to prevent evaporation "rings" and to insure complete wetting of the entire inner surface of the tube. Settling or layering of the organisms is also prevented. Animal inoculation as well as cultural tests are used to determine the efficiency of the various mercurials with this technique.

It is to be concluded from the results of the tests described that none of the mercurials examined can be depended upon to sterilize instruments, when spore-forming organisms are present. In the case of *Clostridius tetani* none of the mercurials rendered these spores non-infectious after twenty-four hours' contact. The spores were still capable of infecting the mice and producing tetanus. The spores of the "clostridium septique" and *Bacillus anthracis*, however, could be rendered non-infectious in certain instances. That is, although it was possible to recover these organisms in cultural experiments, they did not produce an infection when injected into animals.

ROUTINE TESTING OF DISINFECTANTS AND THE SCIENTIFIC ATTITUDE. *C. M. Brewer*, Food and Drug Administration, D. C.

In routine biological tests, even those with as simple a technique as ordinary disinfectant and antiseptic methods, non-uniform and unexplained results are frequently encountered. Officials in the Food and Drug Administration have an unusual opportunity of observing the large number of workers experiencing difficulty in obtaining satisfactory results. It is impossible in the description of routine methods to point out the serious influences which minor and apparently innocuous deviations from prescribed technique may have. When technicians are entrusted with the responsibility of performing routine biological tests, they should be under the supervision of scientifically-minded and broadly trained persons: Examples were cited.

Too complete filtration of the subculture media, in one instance, pre-

vented proliferation of small numbers of test-organism; and illustrated the importance of recognizing the types of death-curves obtained in disinfecting testing as well as the phenomenon of allelocatalysis. In another case, the *resistance of the test-organism* was affected by reducing the diameter of the culture tubes by 5 mm., which reduced the resistance of the organism. This brought out the importance of the surface-area-volume ratio and the necessity of a proper accessibility of oxygen for obtaining resistance of the test-culture.

A recently proposed standard of technique for sporicidal methods to be used for evaluating chemical sterilizers of surgical instruments was cited as a flagrant example of unscientific methods.

HISTORICAL APPROACH TO THE STUDY OF ANTIBACTERIAL ACTION. *Justina Hill*, Johns Hopkins Hospital.

Man's empirical use of antibacterial agents far preceded his knowledge of micro-organisms. This is exemplified by primitive methods of preserving food, by Egyptian mummification, by the Hindu use of copper vessels for water and by some of the surgical techniques of the Hippocratic corpus. Even from the strange decoctions of herbal medicine true antiseptics may be recovered. We now use alcohol where the ancients used wine, and acetic acid where they used vinegar, but the active principles were early recognized. Clinically, establishment

of the fact that gunpowder itself was not poisonous and Paré's accidental discovery that wounds did not have to be treated with boiling oil opened the long road towards a more rational therapy against local infection. The modern era of testing antibacterial drugs began with Sir John Pringle when in 1750 he presented the Royal Society his method for evaluating antiseptics. This included the use of a relatively standard medium, controlled temperature conditions and the establishment of a unit for comparison, sea salt. Within the bacteriological era it must not be forgotten that Lister was an experimental bacteriologist, constantly studying antiseptic substances in tests "conducted in the early morning hours while most men slept in bed." In this country we are proud to remember the work of Sternberg, which he started between Indian fights at Walla Walla in 1878. From Koch's 1881 paper to the present phenol coefficient tests progress has been direct. We now need in part (1) an accurate method for testing the bacteriocidal action of highly bacteriostatic drugs, such as the mercurials (2) a standard bacteriostatic method comparable in inoculum and in as many ways as possible with the bacteriocidal test, in order that bacteriocidal-bacteriostatic ratios may be determined, (3) more agreement in regard to amount of protein to be used, possibly whole blood for drugs intended for clinical use and (4) standard methods for testing the action of drugs in wounds and on the skin.